The Role of Ezh2 in Neural Crest Development

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1 Summary

Stem cells are a key requisite for multi cellular organisms. In multi cellular organisms different stem cell populations create a diversity of specialized cells during development and persist throughout adulthood. The key features of stem cells are their self-renewal capacity and the potential to differentiate into many cell types. Extensive research is in progress to understand the intrinsic and extrinsic factors that regulate the self-renewal behavior and fate restrictions of stem cells.

Neural crest stem cells are a transient population during development that delaminate from the tip of the dorsal tube during its closure, migrate throughout the body to distinct locations and differentiate into a variety of different cell types. Neural crest cells build up glial and neuronal cells of the peripheral nervous system, melanocytes, chondrocytes and osteocytes. Since the fate acquisition of neural crest cells is dependent on their level of emigration from the neural tube, comprising cranial neural crest cells in anterior regions of the embryo and trunk neural crest cells from more posterior locations, these different fate acquisitions have to be differentially regulated. The capability of cranial neural crest cells to build up the chondro- and skeletogenic elements of the craniofacial regions evolved in gnathostomata and allowed a predatory lifestyle due to the development of jaws.

This thesis investigates the role of enhancer of zeste homolog 2, a polycomb repressive complex 2 member in neural crest development. Interestingly, it became apparent that cranial neural crest cells are dependent on enhancer of zeste homolog-2, rather than trunk neural crest cells. The differentiation of trunk neural crest cells in neural derivatives is not impaired, whereas the differentiation of cranial neural crest cells in mesenchymal derivatives is blocked. The blockage of craniofacial chondro- and skeletogenesis comes from the de-repression of Hox

genes in NCCs. In mammals that evolved a neural crest, enhancer of zeste homolog-2 regulates additionally and later in development the repression of Hox genes that otherwise would block chondro- and skeletogenesis. Therefore we observe upon conditional deletion of enhancer of zeste homolog-2 the complete loss of cranial neural crest derived craniofacial structures.

2 Zusammenfassung

Stammzellen sind eine Vorraussetzung für die Entstehung von Mehrzellern. Verschiedene Stammzellpopulationen generieren die Vielzahl spezialisierter Zellen während der Entwicklung von Mehrzellern und selbst in adulten Lebewesen sind noch Stammzellpopulationen zu finden. 2 Schlüsselelemente von Stammzellen sind die Möglichkeit zur Selbsterneuerung und das Potential in verschiedene Zelltypen zu differenzieren. Viel Forschungsaufwand wird betrieben um die intrinsichen und extrinischen Faktoren, die das Selbsterneurungspotential und die Multipotentialität regulieren, zu verstehen.

Neuralleistenstammzellen sind eine während der Entwicklung vorübergehend vorkommende Population von Stammzellen. Diese Neuralleistenstammzellen emigrieren von der dorsalen Seite des Neuralrohrs, während es sich in der Entwicklung schliesst. Sie wandern auf festen Routen durch den Embryo und differenzieren an spezifischen Zielorten in verschiedene Zelltypen. Die Neurallleistenzellen bilden die neuronalen und glialen Zellen des peripharen Nervensystems, sie differenzieren in Pigmentzellen der Haut, Knorpel- und Knochenzellen. In welchen Zelltypus die Neuralleistenzellen differenzieren ist abhängig von dem Ort von dem sie aus dem Neuralrohr emigrieren. Craniale Neuralleistenzellen emigrieren von vorderen Positionen im Embryos aus, wogegen Rumpfneuralleistenzellen eher aus den caudalen Regionen emigrieren. Das Potential der cranialen Neuralleistenzellen Knorpel und Knochen des Kopfes aufzubauen entwickelte sich evolutiv in Gnathostomen und erlaubte es diesen durch die Entwicklung eines Kiefers einen predatorischen Lebensstil anzunehmen.

Diese Arbeit untersucht die Rolle von „Enhancer of zeste homolog-2“, einem Faktor der im „Polycomb repressive complex 2“ integriert ist, in der

Neuralleistenentwicklung. Interessanterweise wurde klar, dass die cranialen Neurallleistenzellen auf Enhancer of zeste homolog-2 angewiesen sind, wogegen die Rumpfneuralleisten nicht affektiert waren. Die Differenzierung der Rumpfneurallleistenzellen in neurale Derivate ist nicht beeinträchtigt, allerdings ist die Differenzierung der cranialen Neuralleistenzellen in die mesenchymalen Derivate blockiert. Diese Blockierung rührt von der Üeberexprimierung von Hox-Genen in Neuralleistenzellen. In Säugetieren reprimiert enhancer of zeste homolog-2 zusätzlich und später in der Entwicklung die Hox Gene, was sonst eine Blockierung der Neuralleistenzellen Differenzierung in die skeletalen Elemente des Kopfes zur Folge hätte. Deswegen sehen wir, aufgrund der konditionellen Deletion von enhancer of zeste homolog-2 in Neuralleistenzellen, das völlige Fehlen der Knorpel und Knochenstrukturen im Schädel, die normalerweise von cranialen Neuralleistenzellen aufgebaut werden.

3 Introduction

3.1 The neural crest

The “neural crest” (NC) was first described by the Swiss Histologist His in 1868 who described a layer of cells between neural ectoderm and non-neural ectoderm as “Zwischenstrang” meaning “in-between-strip” or intermediate cord. More recently, it has already been proposed to call the NC the fourth germ layer, along with endoderm, mesoderm and ectoderm due to the broad potential of neural crest cells (NCC) (Hall 1998; Hall 2000). NCCs get specified in the neural tube and undergo an epithelial to mesenchymal transition to become migratory. The process of emigration of NCCs follows the rostral caudal gradient of neural tube closure during neurulation. After epithelial to mesenchymal transition, NCCs migrate via distinct pathways to their target sites and differentiate into a plethora of cell types. They give rise to neurons and glia cells of the peripheral nervous system, pigment cells of the skin (melanocytes) and cartilage and bone of the head. All these processes are tightly controlled and regulated by extracellular growth factors, intracellular signaling cascades, transcription factor activation/repression and epigenetic modulations (Knecht and Bronner-Fraser 2002; Gammill and Bronner-Fraser 2003; Crane and Trainor 2006; Dupin, Creuzet et al. 2006; Prasad, Sauka-Spengler et al. 2012). The NC is a vertebrate invention; therefore organisms like worms or flies lack it. In phylogenetic groups closer to vertebrates, cephalochordate (e.g. amphiouxus) and even vertebrates, like agnatha (e.g. lamprey) start to express NC specifier and regulatory genes and agnatha already have migratory NCCs, but they lack a jaw. But these species partially have already the regulatory elements and gene expression patterns that later on will become important for NC development (like

homeobox containing (*Hox*) genes and nested distal-less (*Dlx*) expression). The gnathostomes (including e.g. fish, chick and mouse) are the first jawed organisms to appear in evolution. And jaw development was a critical switch in lifestyle, since these organisms became active predators, what facilitated vertebrate radiation into new environments. On the cellular level important changes are the repression of mandibular and maxilliary *Hox* gene expression and the occurrence of nested *Dlx* expression, which allowed craniofacial evolution and ossification of facial elements. The regulatory elements that regulate and fine-tune jaw development were partially duplicated from pre-existing regulatory elements and/or required additional regulatory mechanisms (Gans and Northcutt 1983; Trainor, Melton et al. 2003; Hall and Gillis 2012).

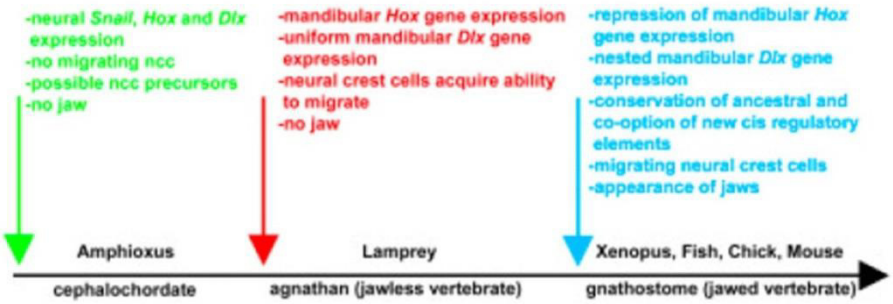

Figure 1: Appearence of the neural crest in evolution

Already cephalochordates show expression of *Snail*, *Hox* and *Dlx* genes but lack a migratory neural crest. Agnathans have a migratory neural crest, but show Hox expression in mandible, naming them jawless vertebrates. The gnathostomes developed a migratory neural crest and the loss of Hox expression in the mandible allowed the development of jaws. Adapted from Trainor, 2003.

3.1.1 Neural crest induction

NCCs get induced at the border of neural and non-neural ectoderm therefore NCCs can be seen as a third layer of ectodermal cells and sometimes even seen as fourth germ layer (Mayor and Aybar 2001). Bone morphogenic protein (BMP) is expressed throughout the ectoderm during gastrulation and suppresses neural fates. Therefore BMP antagonists, like chordin or noggin have to block BMP activity in presumptive neural tissue (Wilson and HemmatiBrivanlou 1997). Additional morphogens that are involved in neural plate border induction are fibroblast growth factors (FGF) and Wnts. FGF signaling can act in chicken as a neural inducer and is required for neural induction (Streit, Berliner et al. 2000; Knecht and Bronner-Fraser 2002). Further, FGF signaling from the paraxial mesoderm plays a critical role for neural plate border induction, since FGFs are capable to induce a subset of neural crest markers (Monsoro-Burq, Fletcher et al. 2003). Wnt proteins are expressed from non-neural ectoderm at the correct time and are neural plate border specifiers, too (Garcia-Castro, Marcelle et al. 2002). These early events of neural plate border specification from inductive signals, like BMPs, FGFs and Wnts activate a gene regulatory network in neural plate border cells, comprising transcriptional regulators and downstream effectors that cooperatively and sequentially provide NC identity. Neural plate border specifier genes that get upregulated, including Msx1/2, Pax3/7 and Zic, enable NCCs to express neural crest specifier genes, like AP-2, FoxD3, Sox9, Snail1/2 (Sauka-Spengler and Bronner-Fraser 2008; Bronner 2012; Prasad, Sauka-Spengler et al. 2012). Some functions of these genes remain elusive but for example, Snail1/Snail2 expression represses E-cadherin to break up the adherent and tight junctions, conferring the ability to migrate to NCCs (Nieto, Sargent et al. 1994; Carl, Dufton et al. 1999; LaBonne and Bronner-Fraser 2000). Another factor for NC specification is FoxD3. In mice it was shown that FoxD3 maintains self-

renewal and multipotentiality in NCCs (Mundell and Labosky 2011). An additional function of FoxD3 might be of more general nature. FoxD3 is considered to open chromatin and potentiating gene transcription as it was shown for a related protein, namely FoxA (Cirillo, Lin et al. 2002). With these gene expression changes NCCs become capable to undergo epithelial to mesenchymal transition, delaminate from the dorsal tip of the neural tube and start migrating. Migratory NCCs start to express the transcription factor Sox10 and the low affinity receptor p75 (Stemple and Anderson 1992; Kapur 1999; Paratore, Goerich et al. 2001; Kim, Lo et al. 2003).

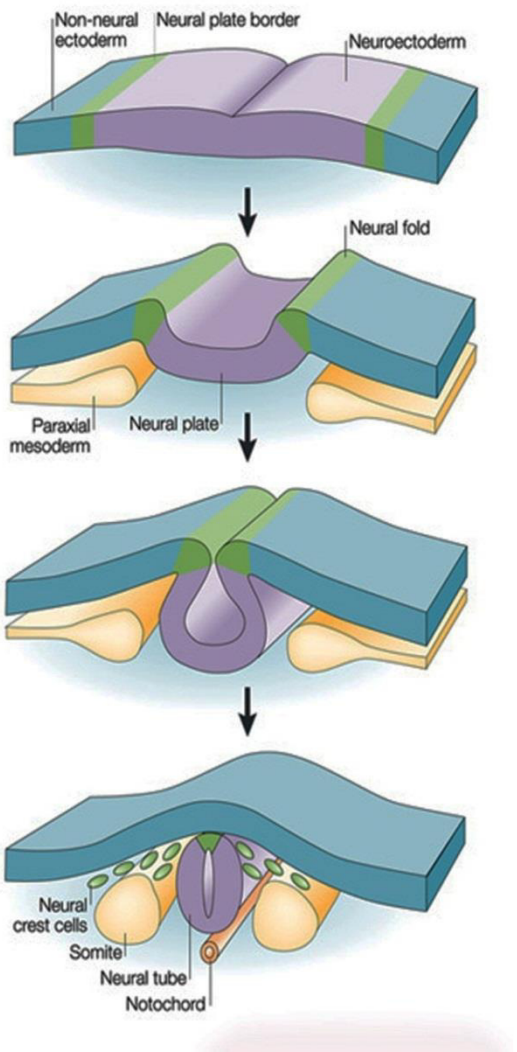

Figure 2: Neural induction and emigration of neural crest cells

Neural crest cells get specified in the neural plate border during neurulation (green area). Surrounding tissue like, neural ectoderm, non-neural ectoderm and paraxial mesoderm secrete factors that specify neural crest cells. After closure of the neural tube neural crest cells undergo an epithelial- mesenchymal transition and become migratory. Adapted from Gammil and Bronner-Fraser, 2003.

3.1.2 Neural crest stem cells

Multipotency and self-renewal capacity are two criteria to define stem cells. Multipotency refers to the fact that a stem cell can give rise to different cell types and self-renewal capacity means the ability to produce a stem cell during cell division (Crane and Trainor 2006). A controversial discussion in the field is the existence of neural crest stem cells (NCSC). Two models are facing each other, both experimentally supported, namely the model describing a multipotent NCSC emigrating and getting fated mainly via environmental signals at target locations. On the other hand the model of pre-specification in the neural tube, showing that the location and time-point of neural crest specification in the neural tube determines the fate of the NCC. The already mentioned criteria for stem cells, like multipotentiality and self-renewal capacity was shown for NCSCs *in vitro* (Sieber-Blum and Cohen 1980; Stemple and Anderson 1992; Kleber, Lee et al. 2005). *In vivo* analysis of NCCs in avian embryos supported the hypothesis of multipotentiality, since single, dye labeled cells, were found in many derivatives build up by the NC, like neurons, glia or melanocytes (Bronner-Fraser and Fraser 1988; Bronner-Fraser and Fraser 1991). Another report showed the quantity of multipotent NCCs in quail neural crest, therefore supporting the NCSC hypothesis again (Trentin, Glavieux-Pardanaud et al. 2004). On the other side is the model of pre-specification in the neural tube, showing that the location and time-point of neural crest specification in the neural tube determines the fate of the NCCs. The pre-specification theory supported by experiments done by Krispin and collegues in chicken via single cell labeling *in vivo*. It was proposed that pre-specification in the neural tube determines the fate of NCCs building up distinct derivatives just in a temporal manner, via migrating dorso-ventrally for neural derivatives and dorso-laterally for non-neural tissues, like melanocytes (Krispin, Nitzan et al. 2010).

NCSC-like cells can even be isolated from post-migratory targets of NCCs. Studies isolating cells from sciatic nerves, dorsal root ganglia (DRG), gut or skin found cells that resemble NCSCs (Morrison, White et al. 1999; Bixby, Kruger et al. 2002; Delfino-Machin, Chipperfield et al. 2007). But post-migratory NCSCs have to adapt to their new environment, resulting in a change of growth factor requirement and/or receptor expression or changes in division mode (White, Morrison et al. 2001; Falk and Sommer 2009; Fuchs, Herzog et al. 2009). And there are reports claiming that NCSC-like cells can be even isolated from adult tissues, like bone marrow or skin (Wong, Paratore et al. 2006; Nagoshi, Shibata et al. 2008).

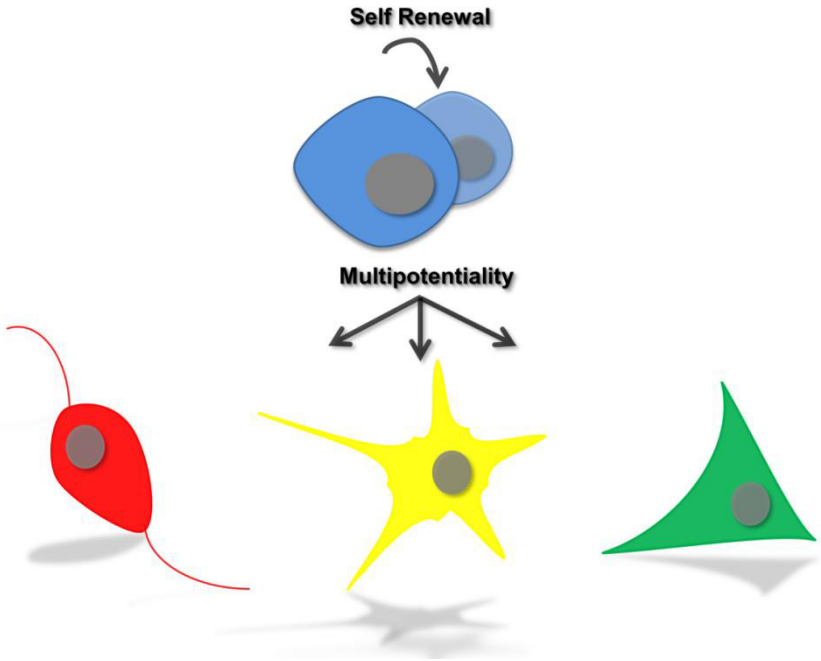

Figure 3: Features of stem cells

The two main features of stem cells. Self-renewal generates two identical daughter cells and multipotentiality refers to the potential of stem cells to differentiate into multiple and different cell types.

3.1.3 Instructive factors for NC fates

In vitro and *in vivo* studies revealed several molecules that act on NCSCs and promote differentiation. These environmental signals are of instructive nature, directly promoting certain fates. Transforming growth factor-beta (Tgf-β) promotes a mesenchymal cell fate of NCCs *in vitro,* mediating the switch of NCSCs to mesenchymal progenitor cells (MPC) that populate the branchial arches (BA) (Shah, Groves et al. 1996; John, Cinelli et al. 2011). *In vivo* Tgf-β receptor ablation in NCCs causes cleft palate and calvarias defect, additionally it regulates self-renewal of midbrain neural stem cells (Ito, Yeo et al. 2003; Falk, Wurdak et al. 2008). When Tgf-β is conditionally ablated in NCCs the defects of DiGeorge syndrome is resembled in mice (Wurdak, Ittner et al. 2005). This indicates an instructive role for Tgf-β signaling in NCCs for adoption of mesenchymal fates. Another report shows the need of NCCs for BMP2 to differentiate into autonomic neurons (Morrison, White et al. 1999). BMPs belong to the Tgf-β superfamily, showing another role for these molecules in NC development. Depending on the context, members of the Tgf-β superfamily promote different cell fates and can even lead to apoptosis (Hagedorn, Suter et al. 1999). The complexity and redundancy of the system *in vivo* is exemplified by work of Buchmann-Moller and colleagues who showed that conditional ablation of Smad4, a downstream target of canonical BMP signaling, in NCCs doesn't lead to neuronal or smooth muscle defects (Buchmann-Moller, Miescher et al. 2009). The glial lineage of NCCs can be promoted with neuregulins (Shah, Marchionni et al. 1994; Jessen and Mirsky 2005). A factor exemplifying the context and time dependent need and function of growth factors, is Wnt1. Already described as a molecule needed for neural plate border induction it has additional roles in NC development at later time points. *In vitro* cultures of NCSCs instructed with Wnt1 adopt a sensory neuronal fate. *In vivo* overexpression of β-catenin, a downstream effector of Wnt signaling, results in sensory neurogenesis to the

expense of other lineages (Hari, Brault et al. 2002; Lee, Kleber et al. 2004). Further, *in vivo* ablation of β-catenin, results in loss of melanocytes in mice (Hari, Brault et al. 2002). Therefore, Wnt/β-catenin signaling is needed at two different time points during NC development and has distinct functions (Hari, Miescher et al. 2012). An overview of different instructive growth factors for NCSCs and they cellular fate is given in Figure 4.

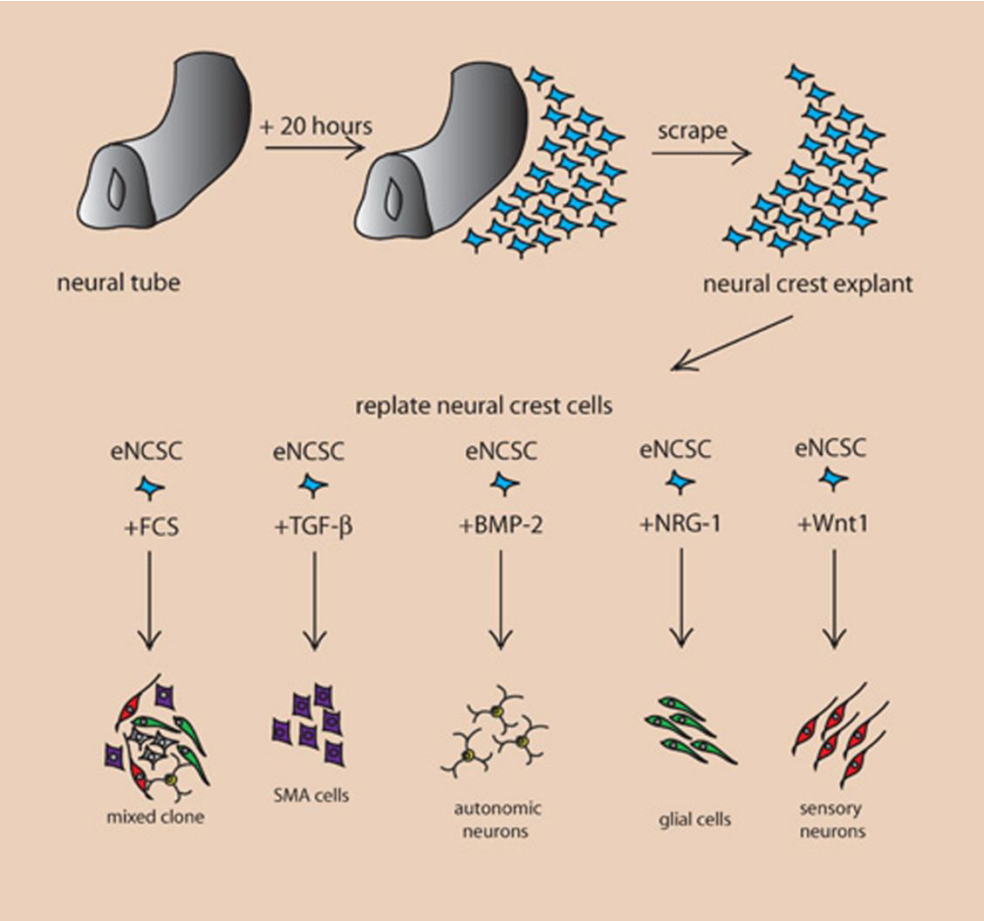

Figure 4: Instructive growth factors for neural crest cell differentiation

With an in vitro culture system for neural crest stem cells it became possible to screen for instructive growth factors. This led to the identification of several instructive growth promoting different fates of neural crest stem cells.
Adapted from Shakhova and Sommer, 2010.

3.1.4 Subpopulations of NCCs

NCC emigration occurs along the neural tube during its closure. Dependent on the rostral-caudal level of emigration, NCCs can be divided into subpopulations. The level of emigration restricts the potential of NCC subpopulations *in vivo*. Cranial neural crest cells (CNCC) emigrate from mid-diencephalic level caudal to somite 4 (rhombomere 7). They have the broadest potential, differentiating into chondrocytes, osteoblasts, neurons, glia cells and melanocytes. CNCCs build up the cranial nerves, melanocytes of the later head region and differentiate into chondrocytes and osteocytes of the facial area. Some bones of the skull are exclusively built up by CNCCs, to others they just contribute. Preliminary structures to the craniofacial derivatives of NCCs are the BAs. Getting established and populated by NCCs from E9.5 onwards, the BAs get remodeled around E12 into their later shape, like maxillary or mandible (Santagati and Rijli 2003; Le Douarin, Brito et al. 2007; John, Cinelli et al. 2011). Another NC subpopulation is the vagal NC. Emigrating NCCs from somites 1-7, therefore partially overlapping with emigration domains of CNCCs, build up the ENS. The colonization of the gut spans approximately from E9.5 to E15.5 establishing the plexus that innervates the gut (Heanue and Pachnis 2007). The cardiac NC, emigrating from the level of the otic placode caudal until somite 3, is part of the cranial neural crest. Emigration domains are completely overlapping with CNCCs. These NCCs build up the outflow tract of the heart, adopting a mesenchymal fate, too (Sieber-Blum 2004). Lastly, trunk NCCs, emigrate from somite 7 to most caudal regions give rise to the PNS. They build up structures, like DRGs, autonomic ganglia (AG), peripheral nerves, they populate the medulla of the adrenal gland and give rise to melanocytes (Le Douarin and Dupin 2012). Their fates are more restricted compared to CNCCs, even though they have skeletogenic potential *in vitro*, they don't differentiate into osteocytes *in vivo* (McGonnell and

Graham 2002; John, Cinelli et al. 2011). Firstly, trunk NCCs differentiate into neurons including neuronal subtypes, like sensory neurons and autonomic neurons (Eng, Lanier et al. 2004; Howard 2005). Secondly, the glial lineage is represented by satellite cells and Schwann cells (Woodhoo and Sommer 2008). And lastly, they give rise to melanocytes in the skin (Thomas and Erickson 2008; Sommer 2011).

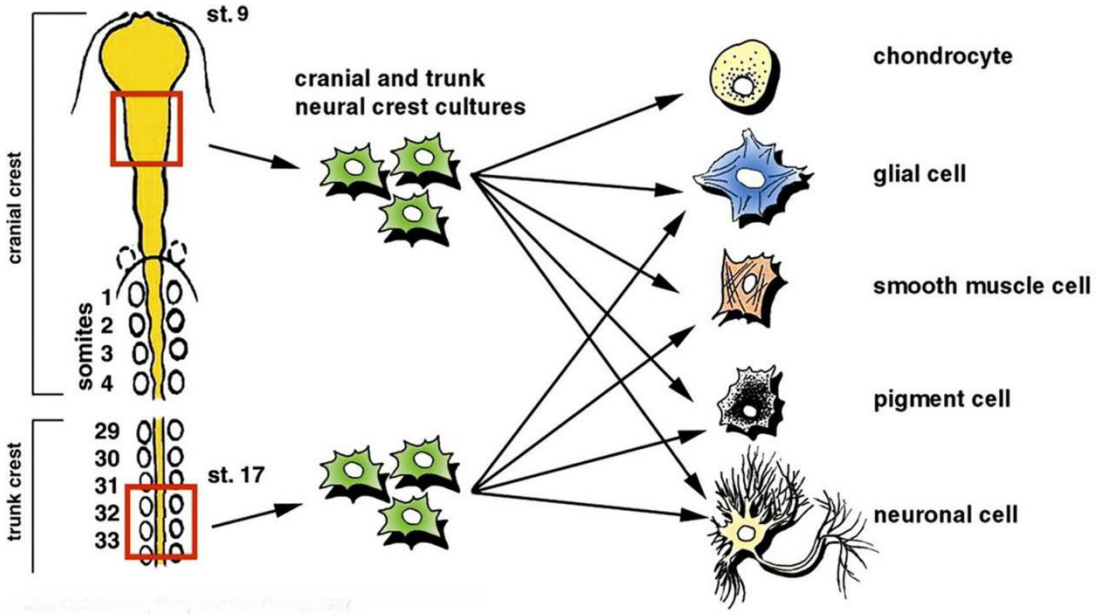

Figure 5: Different levels of emigration for trunk NCCs and CNCCs and their cellular fates

Cranial neural crest cells emigrate from mid-diencephalic levels caudal to somite 4, (rhombomere 7) and these differentiate in to chondrogenic and smooth muscle cells additionally to the fates trunk neural crest cells aquire. Trunk neural crest cells emigrate from somite 7 to most caudal regions and mainly build up the peripheral nervous system (neurons and glia) and melanocytes. Adapted from Abzhanov et al., 2003

3.1.4.1 Cranial neural crest

CNCCs emigrate from the mid-diencephalic level caudal to somite 4 (rhombomere 8) and they contribute to cartilage, bone and connective tissue, comprising additional cell fates in comparison to trunk NCCs (Santagati and Rijli 2003; Le Douarin, Brito et al. 2007). From rostral NCCs the frontonasal processes arise, whereas more posterior CNCCs, mainly emigrating from the rhombomeres of the hindbrain, go through an intermediate step of branchial arch (BA) population to build up cartilage and bone of jaw, middle or neck (Noden 1978; Couly, Coltey et al. 1993; Jiang, Iseki et al. 2002). CNCCs emigrate from domains including the hindbrain. The CNCCs emerging from these domains migrate and populate the BAs. BAs (BA1-BA4) are transient structures during development, getting established in rostral-caudal fashion from E9.5 onwards in mice and the cells get remodeled into terminal structures, like maxilliary or mandible around E12.0 onwards (Chai, Jiang et al. 2000; Jiang, Iseki et al. 2002). The vertebrate hindbrain is patterned uniquely. It is segmented during development into 7 rhombomeres. This segmentation is achieved by a unique *Hox* gene expression code in the rhombomeres, providing spatial information for cranial ganglia or branchiomotor nerves (Trainor and Krumlauf 2001). A factor that establishes the early '*Hox* code' for anterior-posterior patterning in the neural tube is e.g. retinoic acid (RA). Additional factors and more local ones are FGFs or Wnts (Studer, Gavalas et al. 1998; Gavalas and Krumlauf 2000; Irving and Mason 2000; Kiecker and Niehrs 2001). Additionally, the anterior-posterior segmentation of the hindbrain provides migratory information for CNCCs that emigrate from the hindbrain. 3 streams of CNCCs, adjacent to rhombomere2, 4 and 6 can be observed, migrating to and populating BA1, 2 and 3, respectively (Couly and Ledouarin 1988; Serbedzija, Bronnerfraser et al. 1992). CNCCs transpose the Hox code they acquired from the neural tube to the BA mesenchyme (Trainor and Krumlauf 2001). This retained *Hox* gene expression provides anterior-posterior

positional information to the BA mesenchyme. Interestingly, the *Hox* gene expression can be adjusted. All CNCCs in BA1 and more rostral domains should be devoid of *Hox* gene expression to generate skeletal elements of the facial area. But the Hoxa2 expression domain reaches until the border of rhombomere 1 and 2. CNCCs that emigrate from rhombomere 2 express Hoxa2, therefore when they migrate into BA1 they have to silence Hoxa2 expression. Partially, anterior-posterior positional information, in the Hox free domains (BA1 and rostrally), is provided by another homeodomain containing factor, Otx2. Otx2 is expressed in midbrain derived CNCCs, and from these cells the distal mandibular and frontonasal skeletal elements arise, whereas the rhombomere derived Hox free BA1 cells give rise to the proximal parts of these structures (Matsuo, Kuratani et al. 1995; Kuratani, Matsuo et al. 1997). A schematic that illustrates CNCC migration into the BAs (in the scheme named pharyngeal arch (PA)) and their respective Hox code is given in Figure 6.

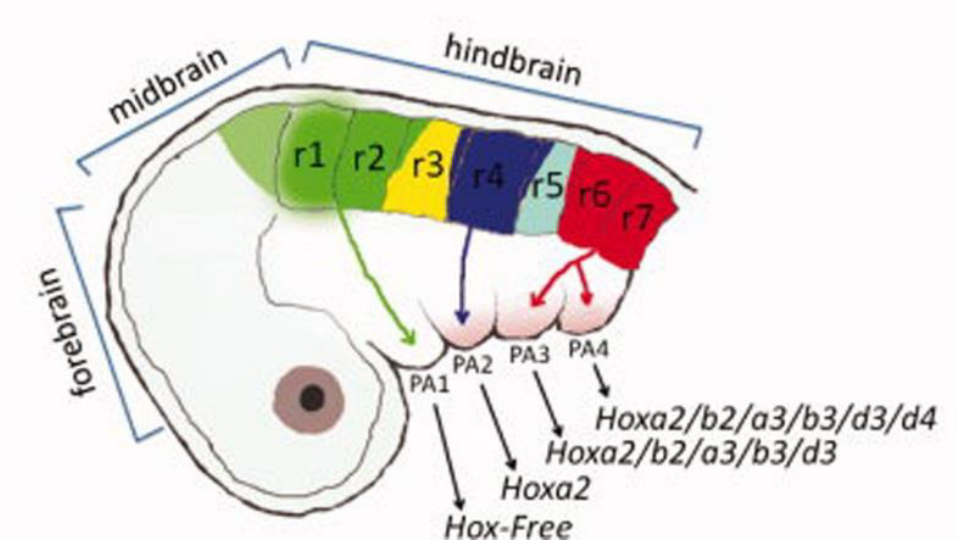

Figure 6: Origin of CNCCs and Hox gene expression in the BAs

Cranial neural crest cells emigrate from the hinbrain which is segmented into 7 rhombomers (r1 – r7). Cranial neural crest cells from r1, r2 and r3 migrate into pharyngeal arch 1 (PA1), part of the cells from r3, r4 and part of r5 migrate to PA2 and cells from r5, r6 and r7 migrate into PA3 and PA4. In the arches cranial neural crest cells have a specific Hox gene expression pattern, having no Hox expression when populating PA1 and more rostral regions, Hoxa2 expression in PA2 and expressing a defined Hox gene code in PA3 and PA4. Adapted from Couly et al. 2002

Further these cells have to get dorso-ventral positional information. Dlx5 and Dlx6 are expressed along this axis in the BA mesenchyme. A knock-out of Dlx5 and Dlx6 results in a homeotic transformation of the lower jaw into an upper jaw (Beverdam, Merlo et al. 2002; Depew, Lufkin et al. 2002; Jeong, Li et al. 2008). The tissue interaction of BA cells and the surrounding cells is another important factor for fate determination of these cells. Intrinsic properties of CNCCs, mediated by Tgf-β signaling that confers a switch from a Sox10-positiv NCSC to the Sox9-positiv, but Sox10-negativ, MPC. These MPCs populate the BAs and enable CNCCs to differentiate into the mesenchymal fates of the NC (John, Cinelli et al. 2011). Tgf-β receptor inactivation in NCCs leads to many defects including cleft palate defects (Wurdak, Ittner et al. 2005). BMP is another signaling molecule that confers specificity to structures derived from CNCCs. Concomitant application of noggin, a BMP antagonist, and retinoic acid transforms the maxillary into a supernumerary fronto-nasal plate (FNP) (Lee, Fu et al. 2001). Another expression source of BMPs is the BA ectoderm. BMP4 in the first place restricts the expression domain of FGF8 in the ectoderm and acts synergistically with FGF8 on CNCCs (Mina, Wang et al. 2002). Further, tissue interactions and signaling molecules are essential and sufficient. FGF8, for example, is expressed by the facial and branchial ectoderm. FGF8, together with BMP4, induce specific gene expression profiles and determines their rostral-caudal polarity in the BA1, but seems to be dispensable for survival of CNCCs in the BAs (Trumpp, Depew et al. 1999; Tucker, Yamada et al. 1999; Shigetani, Sugahara et al. 2002; Hu, Marcucio et al. 2003). Further studies on FGFs and their receptors revealed an important role for FGFs and their respective receptors in osteogenesis, like proliferation, differentiation and even in antagonizing other signaling molecules, like BMPs. FGF2 and FGF4 promote cartilage formation, whereas Fgfr2 regulates stem cell proliferation and Fgfr1 regulates osteogenic

differentiation (Richman, Herbert et al. 1997; Iseki, Wilkie et al. 1999; Sarkar, Petiot et al. 2001; Moore, Ferretti et al. 2002).

All these interactions of intrinsic and extrinsic signals results in the generation of the facial skeleton. But even though CNCCs contribute to a large extend to the facial skeleton they are not the only source of cells building up the bones of the skull. Another source for cells of craniofacial bones is the cranial mesoderm. Vertebrate skulls are assembled by neurocranium (skull vault and base) and viscerocranium (jaws and other BA derivatives). The viscerocranium and the anterior neurocranium are exclusively CNC derived, whereas the posterior skull vault has mesodermal contributions or is of sole mesodermal origin for some bones (Jiang, Iseki et al. 2002; Gross and Hanken 2008; Gitton, Heude et al. 2010).

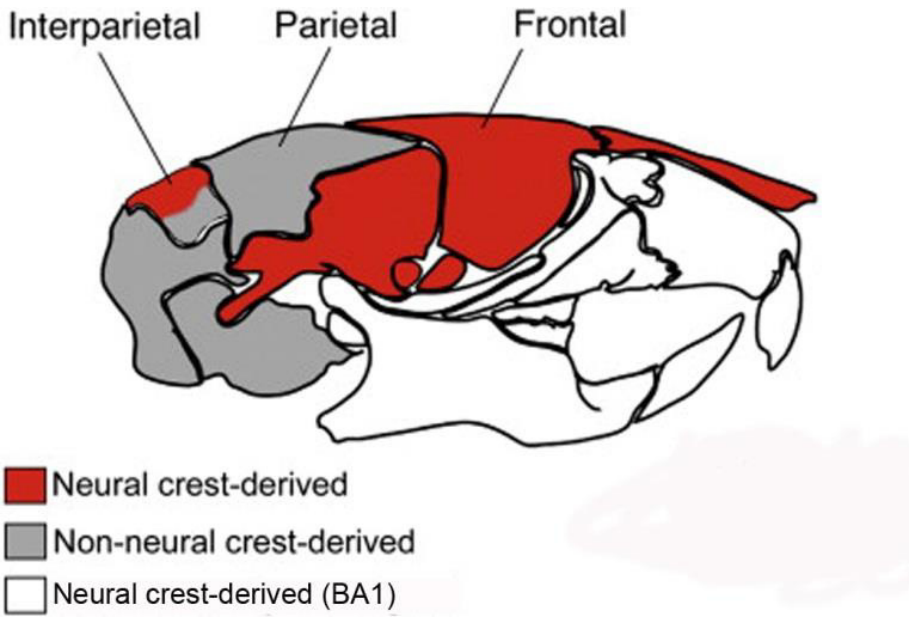

Figure 7: Neural crest derived skull bones

The scheme shows the bones that are cranial neural crest derived (red and white) and which are cranial mesoderm derived (grey). To some bones cranial neural crest cells just contribute, e.g. the medial portion of the interparietal bone. Adapted from Jiang et al., 2002.

3.1.4.2 Trunk neural crest

After emigration from the neural tube from the level of somite 7 to most caudal regions, trunk NCCs migrates in two distinct pathways. The dorso-ventral route and the dorso-lateral pathway (Kuriyama and Mayor 2008). NCCs migrating via the dorso-ventral pathway build up and differentiate into cells from the AG, DRG and when delaminating on a certain axial level of the embryo into chromaffin cells (…). NCCs migrate through the somatic mesoderm or a route between the neural tube and the somatic mesoderm and when migrating through the somite their way is restricted to the anterior somite. These pathway restrictions are mediated by guidance molecules, mainly comprising Ephrins/Ephrin receptors, Semaphorins/Neuropilin and Slit/Robo interactions (Kasemeier-Kulesa, Bradley et al. 2006). Ephrins have a bifunctional role. Early NC is repelled from the dorso-lateral pathway, but at a later developmental stage Ephrins promote melanoblast migration along exactly that pathway (Santiago and Erickson 2002). Another suggested repellent for early NCCs to enter the dorso-lateral pathway is Slit2. Early NCCs express Robo1 and Robo2, suggesting a role of the Slit/Robo interaction on early NCCs migration (Jia, Cheng et al. 2005). Semaphorin 3F, a repulsive ligand, is expressed in the posterior half of each somite, directing NCCs that express the neuropilin2 receptor to migrate through the anterior part of the somite (Gammill, Gonzalez et al. 2006). After arriving at their target location, like AG or DRG, NCCs build up structures and differentiate into specific cell types of the respective structure.

3.2 Epigenetics

The described features of stem cells are intrinsic and extrinsic properties to them. And all these processes underlie at least partially epigenetic regulation. Epigenetics as a term was defined in 1947 by Waddington. The definition underwent a few adjustments during time and is now considered to define all meiotically and mitotically inheritable changes in gene expression without changes in the underlying DNA sequence. Epigenetics include 3 major systems, DNA methylation, RNA interference and histone modifications (Egger, Liang et al. 2004). All three mechanisms together set up an epigenetic state that enables or disables transcription of genes in different regions of the genome.

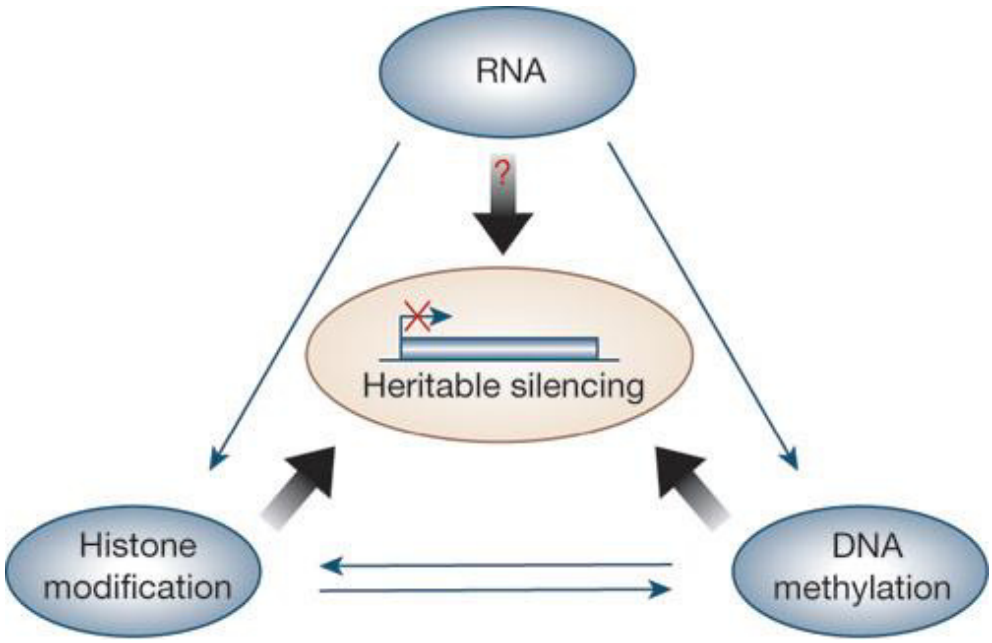

Figure 8: Epigenetic modes

The 3 major epigenetic modes and their considered interactions between each other. All 3 modes result in a heritable gene silencing. Adapted from Egger et al., 2004

A first distinction of chromatin types can be made by histological examination, dividing the genome into euchromatin and heterochromatin. Whereas euchromatin is the sparsely packed chromatin, considered to harbor transcriptionally active genes, the heterochromatin is the densely packed chromatin, containing silent genomic regions (Gaspar-Maia, Alajem et al. 2011). The proportions of euchromatin and heterochromatin are changing during differentiation, with ESCs containing barely heterochromatin. As differentiation progresses the cells silence more and more genomic regions and increase with that the proportion of heterochromatin (Ahmed, Dehghani et al. 2010). Other reports showed the linkage of epigenetic marks to the sort of chromatin, e.g. are histone 3 lysine 9 (H3K9) and histone 3 lysine 27 (H3K27) methylation rich on silent genes and therefore found in heterochromatin, opposed by histone 3 lysine 4 (H3K4) trimethylation standing for active genes present in euchromatin (Meshorer, Yellajoshula et al. 2006; Bhaumik, Smith et al. 2007; Hawkins, Hon et al. 2010). This reflects the intermingled functions of epigenetic modifications and the complexity of how gene transcription is regulated on a non DNA level.

3.2.1 Histone modifications

A nucleosome is defined as a unit of 146bp of DNA wrapped around an octamer of dimers of histones H2A, H2B, H3 and H4. A linker region of DNA between two nucleosomes is associated with histone H1. A nucleosome is the repetitive, single unit that builds up chromatin. N-terminally, polypeptide chains extend from the histone core region. These histone tails are subject to posttranslational modifications (Olins and Olins 2003). Histone modifications include acetylation of lysines, methylation of arginines and lysines and phosphorylation of serines and threonines among others. Several modifications are present simultaneously on a histone tail and their overall presence influence higher order of chromatin or specifically modulate gene transcription (Berger 2007; Kouzarides 2007; Turner 2007).

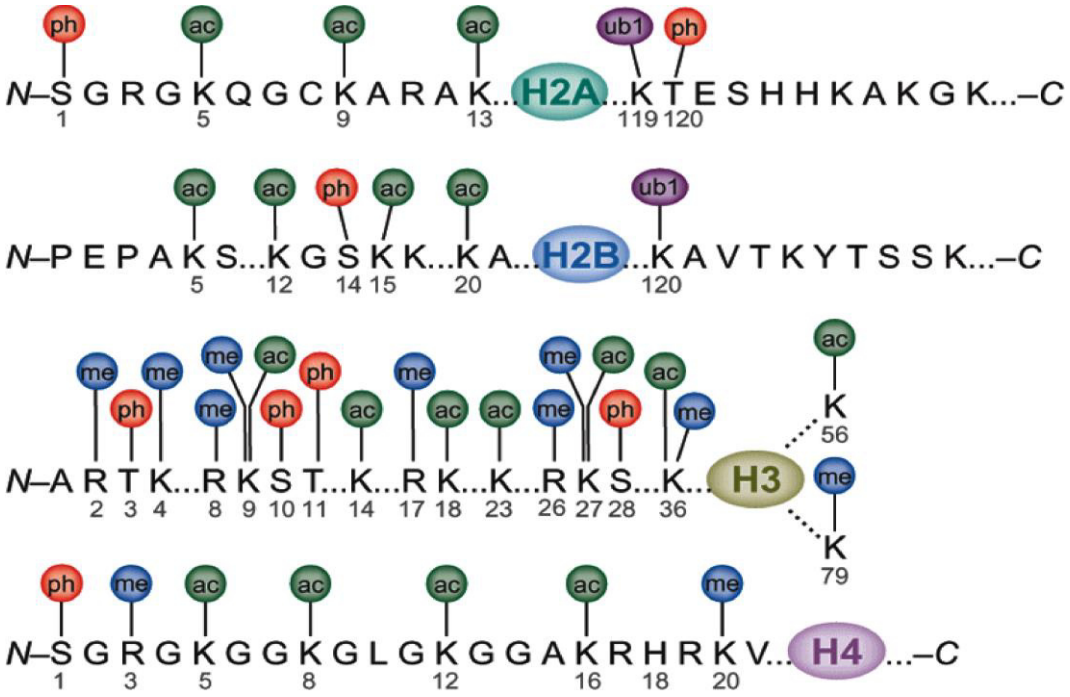

Figure 9: Histone modifications

Schematics of possible modifications that can be placed on the histone tails. All 4 histone proteins are subjects to modifications, like phosphorylation, acetylation methylation and ubiquitination. Adapted from Kouzardis et al., 2008

Just lysine residues of histones are acetylated and acetylation is correlated with transcriptional activity. The acetylation of lysine residues is thought to neutralize the positive charge of lysines, alters the histone-DNA interactions and with that opens the chromatin structure, making the DNA accessible for transcription factors. Acetylation and deacetylation of lysine residues, is catalyzed by histone acetyl transferases (HATs) and histone deacetylases (HDACs), respectively. Due to its ability to open chromatin many transcriptional complexes, like p300/CBP or NCoR/SMRT have acetylation or deacetylation activity, dependent on their own function on transcription (Shahbazian and Grunstein 2007).

A prominent example for histone phosphorylation is the phosphorylation of serine 10 or 28 on H3. The serine residues get phosphorylated just during M-Phase of the cell cycle and serve as a read-out for mitotically active cells in M-phase. The phosphorylation id mediated by the Aurora kinases. An advantage is that PHH3 positive cells are easily distinguishable from apoptotic cells. Immunohistochemistry with PHH3 antibodies is already used in clinics due to its specificity and even fulfills WHO standards (Ribalta, McCutcheon et al. 2004). But other amino acid residues are also phosphorylated during cell cycle, like H2A serine 1, too (Barber, Turner et al. 2004).

Histone methylation was found on arginines and lysines of histones 3 and 4. For histone lysine methylation, just found on amino acid residues of H3 and H4, six lysines are targets of methylation. Lysine 4, 9, 27, 36 and 79 of histone 3 and lysine 20 of histone 4 can be mono-, di- or trimethylated. Contrary to acetylation, lysine methylation can be either an activating or repressing signal, depending on the lysine residue that gets modified. Whereas H3K4me3, H3K36me3 and H3K79me3 are active marks, the methylation of the other lysine residues would result in a transcriptional repression. The arginine's 2, 8, 17 and 26 of H3 or arginine 3 of H4

can just be mono- or dimethylated and the transcriptional outcome depends on the methylated residue, as well. Enzymes of the PRMT and CARM members are the known methyltransferases (Zhang and Reinberg 2001; Volkel and Angrand 2007).

Almost all of these modifications on amino acid residues share a common feature. They are active processes, requiring enzymes that catalyze the deposition of chemical groups to the amino acid residue. Therefore enzymes exist that remove the groups again if needed, e.g. during differentiation when gene transcription profiles change due to cell specification. This process is dynamic and e.g. responsible for the increasing proportions of heterochromatin towards terminal differentiation that reflects the ongoing deposition of repressive marks (Sparmann and van Lohuizen 2006; Turner 2007).

Chromatin Modifications	**Residues Modified**	**Functions Regulated**
Acetylation	**K**-ac	Transcription, Repair, Replication, Condensation
Methylation (lysines)	**K**-me1 **K**-me2 **K**-me3	Transcription, Repair
Methylation (arginines)	**R**-me1 **R**-me2a **R**-me2s	Transcription
Phosphorylation	**S**-ph **T**-ph	Transcription, Repair, Condensation
Ubiquitylation	**K**-ub	Transcription, Repair
Sumoylation	**K**-su	Transcription
ADP ribosylation	**E**-ar	Transcription
Deimination	**R > Cit**	Transcription
Proline Isomerization	**P-cis > P-trans**	Transcription

Table 1: Different histone modifications

Overview over the different histone modifications found up to date. Residues that are modified are given in the 1 letter amino acid code and the functional outcome is listed. Adpated from Kouzarides, 2007.

3.2.1.1 The polycomb repressive complex 1 and 2

In *Drosophila*, polycomb group genes (*PcG)* genes were identified to repress *Hox* genes during developmental patterning. Mutations of *PcG* members results in incorrect de-repression of *Hox* genes and disrupts correct spatial and temporal patterning during embryonic development. The phenotype was named polycomb, since body segementation was posteriorized and showed additional sex combs (Ringrose and Paro 2004; Sparmann and van Lohuizen 2006; Alexander, Nolte et al. 2009). In vertebrates, *Hox* gene mis-expression, caused by *PcG* mutations, lead among others to skeletal phenotypes (Akasaka, Kanno et al. 1996; Lorente, Marcos-Gutierrez et al. 2000). The *PcG* genes encode two distinct complexes that both are able to remodel chromatin, namely polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2).

PRC1 is composed of the core components Ring1A/B, Bim1, Cbx and Phc. Ring1A/B are E3 ubiquitin ligases and therefore the catalytic subunit of PRC1 catalyzing the ubiquitination of histone H2A at lysine 119. H2AK119ub1, a repressive mark, inhibits gene transcription (Levine, Weiss et al. 2002; Wang, Wang et al. 2004). The Cbx protein of PRC1 has a chromodomain that recognizes the H3K27me3 mark set by PRC2. One can assume that these events might be causal to each other, but reports already showed that chromatin compaction occurs even without ubiquitination activity of Ring1B and partially, ubiquitination of H2A119 takes place without recruitment of PRC1 by H3K27me3 (Eskeland, Leeb et al. 2010; Tavares, Dimitrova et al. 2012).

The PRC2 is composed of 4 core subunits Ezh2, Eed, Suz12 and RbAp46/48 (Margueron and Reinberg 2011). Ezh2 with its SET-domain is the catalytic subunit of PRC2 that catalyzes the mono-, di- and trimethylation of H3K27 (H3K27me1,

H3K27me2, H3K27me3) (Shen, Liu et al. 2008; Schuettengruber and Cavalli 2009). Eed is a linker between histone H3 substrates and Ezh2 (Tie, Stratton et al. 2007) and Suz12 is needed to stabilize Ezh2 and for nucleosome recognition (Pasini, Bracken et al. 2004; Nekrasov, Wild et al. 2005). *In vitro* experiments showed that RbAp46/48, a histone binding protein, binds to PRC2 and enhances its enzymatic activity (Cao and Zhang 2004). Straight knock-outs of *Eed*, *Suz12* or *Ezh2* in mice showed similar phenotypes, pointing to the coordinated function of the PRC2 complex members. Mice lacking one of these proteins die around gastrulation. Therefore it is not possible to use them to study *in vivo* functions of PRC2 members at later developmental stages (Faust, Schumacher et al. 1995; O'Carroll, Erhardt et al. 2001; Pasini, Bracken et al. 2004). PRC2 acts as a transcriptional repressor, since the H3K27me3 mark is found mainly at silenced promoters (Mikkelsen, Ku et al. 2007; Schuettengruber, Chourrout et al. 2007). In mammals, PRC2 is involved in repressing developmental regulators in mouse embryonic stem cells (mESCs) and PRC2 regulates the proliferation and differentiation of stem cells (Boyer, Plath et al. 2006; Margueron and Reinberg 2011). PRC2-Ezh1 is an additional mammalian PRC2 complex with Ezh1 substituting for Ezh2. The PRC2-Ezh1 has a similar molecular weight as PRC2-Ezh2 but exhibits lower methyltransferase activity. The way to repress gene transcription is different for both complexes. Knockdown of Ezh1 does not change global H3K27me3 levels but PRC2-Ezh1 is able to condensate chromatin. The targets of Ezh1 and Ezh2 are just partially overlapping and only a double knock-out of *Ezh2* and *Ezh1* abolishes H3K27me3 completely in ESC, resulting in a de-repression of H3K27 target genes (Margueron, Li et al. 2008; Shen, Liu et al. 2008). Additional information for redundant functions of Ezh1 and Ezh2 comes from work in skin development. Ezh2, abundant in epithelial stem cells, declines during differentiation and Ezh1 expression increases in terminal

differentiated layers, indicating that Ezh1 overtakes Ezh2 function in more differentiated tissues (Ezhkova, Pasolli et al. 2009; Ezhkova, Lien et al. 2011).

An unsolved question is how PRCs get recruited to their target genes. In *Drosophila* the search was already narrowed down. DNA consensus sites for binding of PHO and PHO-like, both zinc finger proteins, are essential for the recruitment. This complex, called PHO-RC interacts physically with subunits of PRC2 and PRC1. PHO mutants de-repress *Hox* genes, because of PRC1 and PRC2 missing at *Hox* gene PREs (Wang, Brown et al. 2004; Mohd-Sarip, Cleard et al. 2005; Klymenko, Papp et al. 2006). However, up to now no PRE could be identified in mammals. Data points to a more complex regulation, involving many different factors and tissue specific recruitment. Oct4 and/or Yy1 are candidates for recruitment factors of PRCs, but one has to keep in mind their own tissue specific expression. Further, other epigenetic marks like CpG islands are discussed and ncRNAs were suggested for PRC recruitment, too (Squazzo, O'Geen et al. 2006; Rinn, Kertesz et al. 2007; Ku, Koche et al. 2008; Zhao, Sun et al. 2008). An overview about PRC1 and PRC2 and their respective member is given in Figure 10.

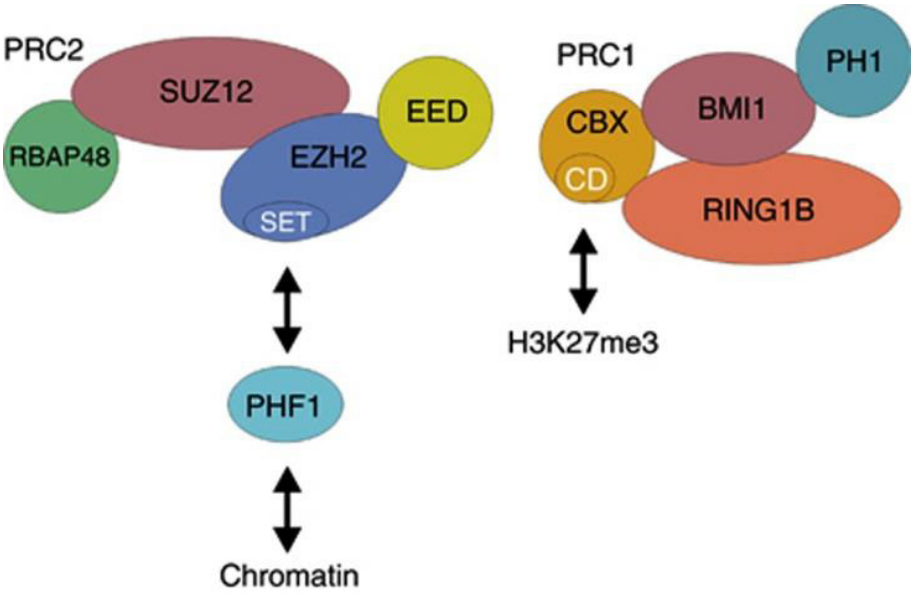

Figure 10: The polycomb repressive complexes 1 and 2

The polycomb repressive complex 2 consists of four core proteins, RBAP48, SUZ12, EED and EZH2 with its SET-domain. Ezh2 activity as methylatransferase requires interactions with the other complex members. PHF1 is not part of the core complex itself, but required for chromatin binding of the complex. PRC1 has 4 core members, as well. PH1, BMO1 CBX, with its chromo-domain and RING1B, an ubiquitin ligase for ubiquitinination of H2AK119. CBX and the chromodomain are recognizing the H3K27me3 mark set by polycomb repressive complex 2. Adapted from Eckert et al., 2010.

3.2.1.2 Ezh2

The gene for enhancer of zeste homolog 2 (Ezh2) lies on chromosome 6 in mice. It spans over ~65 kbps. The protein is encoded from 20 exons and the exons 16-20 encode for the SET-domain of Ezh2. The SET-domain is the catalytic subunit of Ezh2 and with that for the whole PRC2. Ezh2 belongs to the EZH family of SET-domain containing proteins. N-terminally to the SET-domain it has a 15aa cysteine rich residue but no post SET-domain and the protein comprises 2 SANT-domains. The counter-actors of Ezh2 activity are jmjC-domain containing enzymes that de-methylate H3K27me3. Uty, Utx and jmjd3 are known H3K27me3 demethylases (Volkel and Angrand 2007).

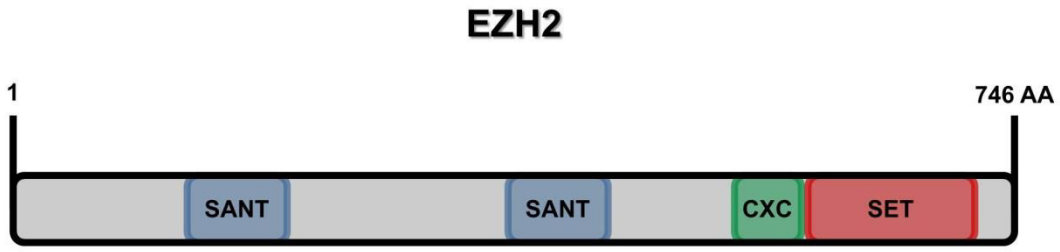

Figure 11: Ezh2 protein with main domains

Schematics of Ezh2 protein structure with 2 SANT domains for interactions with histones, a cystein rich 15 amino acid residues before the SET domain, which is the catalytic subunit of the protein and the whole polycomb repressive complex 2. The Ezh2 protein itself is 746 amino acids in length.

Ezh2 is required in many different cellular processes and a crucial protein in development, as all PRC2 members. Embryos lacking *Ezh2* die during gastrulation (O'Carroll, Erhardt et al. 2001), and ESCs *in vitro* devoid of *Ezh2* expression show differentiation defects, whereas maintenance or self-renewal stays largely unaffected due to compensation of Ezh1 (Shen, Liu et al. 2008). In limb development of mice, *Ezh2* has a crucial role in the anterior-posterior axis specification and proximodistal axis elongation (Wyngaarden, Delgado-Olguin et al.

2011). Another report showed the occupancy of H3K27me3 over the HoxD cluster during mouse tail development (Soshnikova and Duboule 2009). In cortical development it was already shown that Ezh2 is responsible for the switch of neural stem cells from the neurogenic to the gliogenic phase. Interestingly, differentiation was not affected per se (Hirabayashi, Suzki et al. 2009). Another report showed similar results pointing to a role for Ezh2 in the timing of cortical development regulating the timing of the neurogenic phases (Pereira, Sansom et al. 2010). In skeletal muscle cell differentiation, Ezh2 knockdown results in premature differentiation of myoblasts to myotubes, pointing again to a role of Ezh2 as a maintenance factor, like in neural stem cells (Caretti, Di Padova et al. 2005; Juan, Kumar et al. 2009). Further, the H3K27me3 demethylase Utx was described to be involved in the demethylation of muscle specific genes during myogenesis, showing the opposing roles of these counteracting enzymes in muscle cell differentiation (Seenundun, Rampalli et al. 2010). In epidermal differentiation Ezh2 exerts similar functions. Upon knock-out of *Ezh2* in epithelial stem cells, differentiation accelerates and self-renewal is impaired. Again, Ezh2 acts as a maintenance factor for stem cells, keeping them in an undifferentiated state, and further Ezh2 regulates self-renewal in these cells (Ezhkova, Pasolli et al. 2009; Zhang, Bardot et al. 2012). It was directly shown that Ezh2 is a cell cycle regulator, too. Ezh2 regulates the *Cdkn2a* locus and by itself gets regulated by posttranslational protein modifications. Phosphorylation of tyrosine residue 641 of Ezh2 by CDKs is needed to regulate Ezh2 activity during cell cycle and probably to spread the H3K27me3 mark through cell divisions (Chen, Bohrer et al. 2010; Kaneko, Li et al. 2010; Zeng, Chen et al. 2011). Ongoing, Ezh2 and various other PRC2 members are described to be mis-regulated in different cancers, like prostate cancer, lymphoma or glioblastoma. *Ezh2* is a proposed oncogene. The functional outcome differs, but again inappropriate cell state maintenance and/or cell cycle misregulation are two key features of *Ezh2*

misregulation in cancers. Downstream of the pRB-E2F transcription factor pathway, *Ezh2* is associated with cell proliferation, whereas Ezh2 overexpression would lead to hypermethylation of tumor suppression genes (Bracken, Pasini et al. 2003; Pasini, Bracken et al. 2004; Suva, Riggi et al. 2009; Qi, Chan et al. 2012).

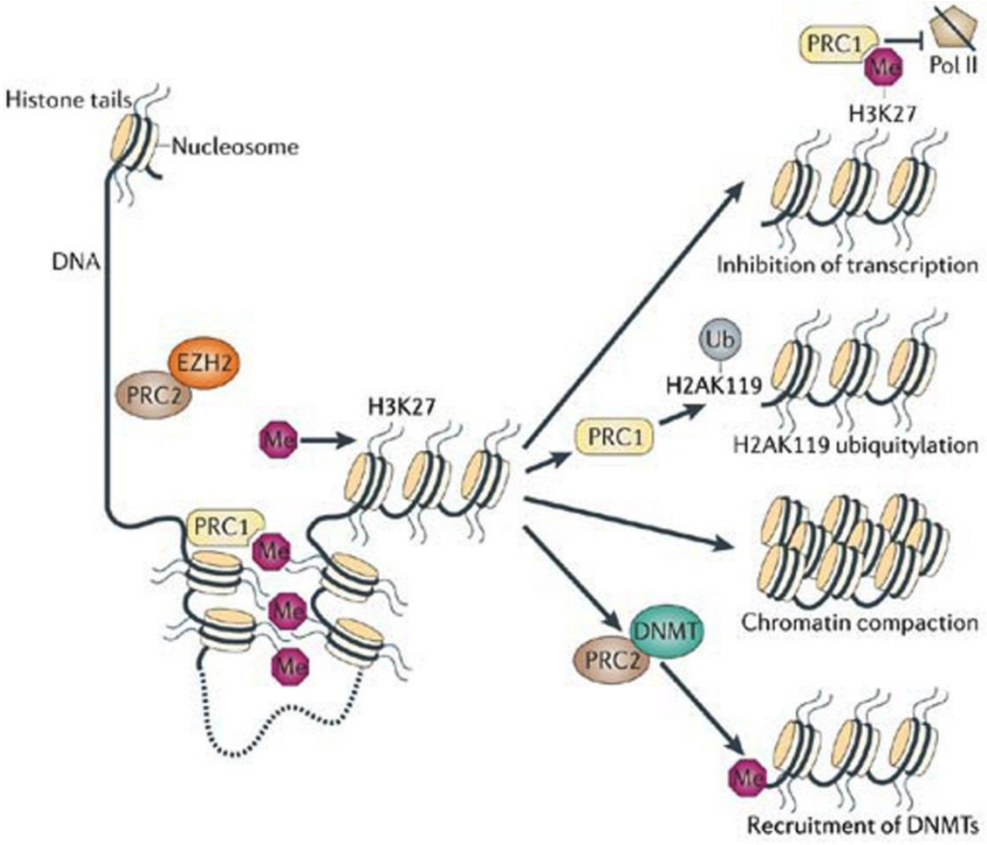

Figure 12: Polycomb repressive complex 2/Ezh2 and subsequent effects

Enhancer of zeste homolog 2 as a subunit of polycomb repressive complex 2 catalyzes the trimethylation of H3K27. This modification can be „read“ by polycomb repressive complex 1, what leads to ubiquitination of H2AK119. Chromatin compaction and repression of gene transcription is the result. Further data indicates the direct interaction of enhancer of zeste homolog 2 with DNA methyltransferases that methylate DNA, another mechanism to repress gene transcription. Adapted from Sparmann and van Lohuizen, 2006.

3.2.2 RNA interference

With the finding of micro RNAs (miRNA) and non-coding RNAs (ncRNA) in nematodes and mammalians, respectively, it became obvious that gene expression can be regulated by RNAs, too (Borsani, Tonlorenzi et al. 1991; Brockdorff, Ashworth et al. 1992; Wightman, Ha et al. 1993). First considered as rare exceptions to already known ncRNAs, like ribosomal RNA or transfer RNA, it became obvious, over the following years, that thousands of ncRNAs are encoded in the genome and that they are involved in almost every biological aspect (Carninci 2009; Jacquier 2009; Mercer, Dinger et al. 2009). A popular example, linked to the PRC2, for ncRNA regulation of gene expression in mammalians is *Xist*. Functionally, *Xist* helps balancing gene dose of X-chromosomal genes between sexes. This X-chromosomal inactivation is mediated by a genomic region called 'X-inactivation center' (*Xic*), encoding for at least 7 ncRNAs (Lee 2009). A 17kbp long transcript from that locus is *Xist*. It acts in a negative feedback loop, inactivating first the locus and subsequently the chromosome it is transcribed from. *Xist* by itself recruits PRC2 resulting in H3K27me3 methylation of the 5' end of *Xist.* Starting from there the whole X-chromosome gets repressed in a *Xist* dependent manner (Zhao, Sun et al. 2008). Another example for ncRNA regulation of gene expression are *Hox* genes. Transcription of non-coding regions of the Hox cluster can regulate the expression of neighboring *Hox* genes. A HOX antisense intergenic RNA (HOTAIR) was found in humans. HOTAIR has a silencing activity, due to its interactions with PRC2 and a complex containing the H3K4me3 demethylase LSD1. The repression of the HOXD cluster and other loci is HOTAIR-dependent (Rinn, Kertesz et al. 2007; Tsai, Manor et al. 2010; Pauli, Rinn et al. 2011).

3.2.3 DNA methylation

Another epigenetic mark that was described to be repressive is the methylation of cytosine residues occurring in CpG dinucleotides. CpG islands, meaning stretches of DNA containing more than statistically expected CG- nucleotide palindromes, were found on more than 50% of the genes in vertebrate genomes and especially around promoters. DNA methylation is an active process requiring DNA methyltransferases (DNMTs). DNMT1, DNMT3A and DNMT3B are described DNA methyltransferases. Since epigenetic modifications are heritable it was thought that DNMT1 would overtake functions in re-methylating one DNA strand after cell division and is therefore called maintenance DNMT. Further studies on the function suggested interactions with other protein complexes refusing DNMT1 to act alone on DNA methylation maintenance. On the other hand, DNMT3A and DNMT3B are de novo methyltransferases (Bird 2002; Cedar and Bergman 2012). An *in vivo* knock-out of any of these enzymes results in embryonic lethality, emphasizing the crucial role of DNA methylation for development of vertebrates (Li, Bestor et al. 1992; Okano, Bell et al. 1999). Contrary, all three DNMTs are dispensable for the self-renewal ability of ESCs *in vitro* (Tsumura, Hayakawa et al. 2006). This data points to roles of DNMTs in specific cellular functions that have to be elucidated further. Again showing the complementary functions of epigenetic modifications, interaction of Ezh2 and DNMTs was already shown, suggesting that Ezh2 action, and with it H3K27 trimethylation, directly controls DNA methylation via recruiting DNMTs (Vire, Brenner et al. 2006). This interlink of Ezh2 and DNMTs was shown in cancer, too (Schlesinger, Straussman et al. 2007).

3.3 The Cre-loxP system

Transgenic mice became a valuable tool for biological research. Especially the *Cre/loxP* system allowed studies in tissue specific stem cells and their progeny. Another advantage is circumvention of early lethality of straight knock-outs of some genes in animals. The *Cre/loxP* system requires the introduction of *loxP* sites next to exons/genes that should be cut out on mice. On the other hand, one needs the Cre-recombinase, an enzyme that recognizes the *loxP* sites with different outcomes dependent on the orientation of the *loxP* sites. Are the *loxP* sites directly repeated the recombination event will result in an excision of the targeted DNA sequence within the *loxP* sites. If they would be inverted, this would result in an inversion of the targeted DNA sequence. In dependency of the promoter used to drive the Cre-recombinase expression, one can control tissue specificity and timing of Cre-recombinase activity and with that the deletion of the gene of interest. Manifold usages are applied today. Firstly, used to delete genes in a spatio-temporal controlled manner, one can also use the *Cre/loxP* system to delete STOP-codon that are inserted before genes, e.g. for overexpression of genes or to express a reporter gene, like *LacZ* for lineage tracing of recombined cells (Sauer and Henderson 1988; Turan, Galla et al. 2011).

4 Material and Methods

4.1 Animals and genotyping

The *Cre-loxP* system (Sauer and Henderson 1988; Turan, Galla et al. 2011) was used to conditionally ablate *Ezh2* in NCCs. Mice carrying a transgene, where 2 exons encoding for the SET-domain of *Ezh2* are flanked by *loxP* sites, are described (Hirabayashi, Suzki et al. 2009) and shown in Figure 13. Homozygous animals (*Ezh2*$^{lox/lox}$) were crossed with animals carrying the *Wnt1*-Cre transgene (Danielian, Muccino et al. 1998) and that are heterozygous for the floxed *Ezh2* allele (*Wnt1-Cre; Ezh2*$^{lox/wt}$). Offspring with the genotype *Wnt1-Cre; Ezh2*$^{lox/lox}$ are termed Ezh2 cko and got compared to their respective littermates with genotypes having either no *Wnt1-Cre* transgene (*Ezh2*$^{lox/lox}$ *or Ezh2*$^{lox/wt}$) or lacking a second copy of the floxed *Ezh2* transgene (*Wnt1-Cre, Ezh2*$^{lox/wt}$) since these animals never showed an overt phenotype and the lastly mentioned served as mating studs with normal life-time expectations. Ezh2 cko mice survived to late developmental stages although they never got born. Time mating's were done o/n and noon on the following day was considered as embryonic day (E) 0.5. In vivo fate mapping of NCCs was done on mice additionally carrying the *R26R-LacZ* allele (Soriano 1999) that gets activated upon Cre mediated recombination to express β-galactosidase.

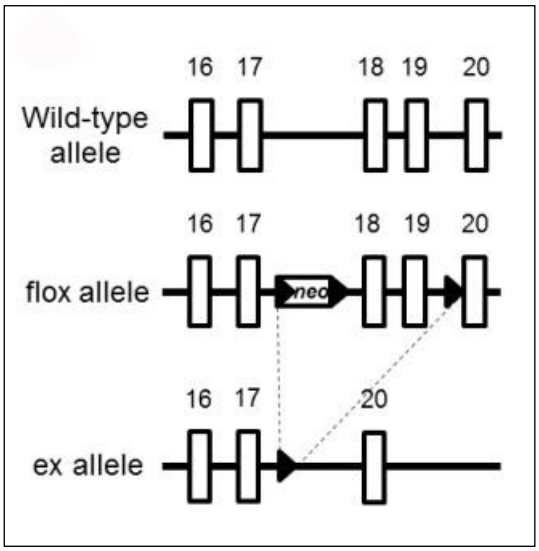

Figure 13: Ezh2 alleles

The exons 16-20 of the wild-type allele of Ezh2 are shown. These exons encode the SET-domain of Ezh2.

The exons 18 and 19 are flanked by loxP sites in the flox allele.

Upon Cre-mediated recombination exons 18 and 19 are deleted and with that the encoded protein lacks a funcional SET-domain.

Genotyping was done by PCR on genomic DNA obtained from tails. The primers used are listed in Table 2.

	Forward primer	Reverse primer
Wnt1-Cre	ATGCCCAAGAAGAAGAGGAAGGT	GAAATCAGTCGCTTCGAACGCTAGA
Ezh2 wt	AAGGCTGTGTACAGGAAACAA	AGTACTCCATACAGGAAACAATC
Ezh2 fl	AAGGCTGTGTACAGGAAACAA	TCACCTTAATATGCGAAGTGGAC
LacZ	GGTCGGCTTACGGCGGTGATTT	AGCGGCGTCAGCAGTTGTTTTT

Table 2: Primers for PCR

4.2 Immunohistochemistry, X-Gal staining and EdU staining

For immunohistochemistry, 14-µm thin cryosections were fixed 10 minutes in 4% formaldehyde in PBS at room temperature, on purpose followed by an antigen retrieval with 10mM citrate buffer, pH 6, for 10 minutes at 110°C in a microwave, then blocked 1 hour in blocking buffer (1% BSA, 0.3% Triton-X100 in PBS) at room temperature and incubated with the respective primary antibody (see Table x) at 4°C o/n. Secondary antibody incubation was done 1 hour at room temperature. DAPI (1:2000) staining was done 5 minutes at room temperature; afterwards the slides were mounted and covered for analysis. Primary antibodies were used as follows: rabbit anti-PHH3 (1:200, Millipore), mouse anti-tyrosine hydroxylase (TH) (1:200, Sigma), mouse anti-Mash1 (1:100, BD Biosciences), rabbit anti-Brn3a (1:2000, a gift from E. E. Turner, Seattle Children's Research Institute, Seattle, WA, USA), mouse anti-Neurofilament 160 (1:400, Sigma), rabbit anti-Neurofilament M (1:200, Chemicon), rabbit anti-Ki67 (1:200, Abcam), rabbit anti-FABP (1:4000, a gift from C. Birchmeier, Max Delbrück Center for Molecular Medicine, Berlin, Germany), rabbit anti-cleaved Caspase-3 (1:200, Cell signaling), rabbit anti-H3K27me3 (1:200, Millipore), rabbit anti-Oct6 (1:200,), rabbit anti-Collagen2a1 (1:200, Acris). Fluorescence conjugated antibodies were from Jackson ImmnunoResearch or Invitrogen. *LacZ* reporter gene expression was detected by incubating the embryos stage dependent (2 to 12 hours) at 37°C in PBS containing 1 mg/ml X-Gal (Applichem), 10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ and 0.02% NP40. Reaction was stopped by placing the embryos in PBS. EdU stainings were done according to the manufacturer's protocols (Invitrogen). EdU was injected 1 hour before euthanization intra-peritoneal of time mated animals at E11.5.

4.3 Primary cell culture isolation (BA1 cells)

BA1 cells were mechanically isolated from E11.5 embryos and kept separately on ice. The BAs were digested in HBSS (Gibco) containing 0.025% Trypsin (Gibco) and 0.35 mg/ml collagenase type I (Worthington) for 15 minutes at 37°C. The reaction was stopped by adding 10% FCS (Gibco) to the digestion mixture. Cells were spinned down for 5 minutes with 1200 rpm and re-suspended in buffers applicable for further experiments and stored until genotyping was done (see also Figure 14).

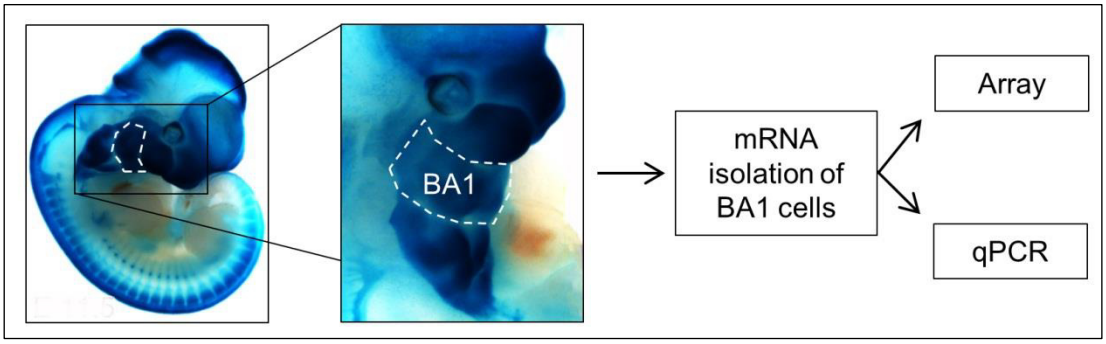

Figure 14: Worklow of BA1 cell isolation from E11.5 embryos

4.4 Cell cycle FACS analysis

Wnt1-Cre; Ezh2$^{lox/lox}$ and *Ezh2*$^{lox/lox}$ BA1 cells, isolated as described in 4.3, were taken up in 500 µl PBS incubated 15 minutes on ice containing 1.25 mM 5-AAD (Sigma), washed twice in PBS and then analyzed using a FACS Canto II flow cytometer (BD Bioscience). FlowJo software (Tree Star) was used to analyze the data.

4.5 Quantitative RT-PCR and ChIP-q-PCR

For mRNA isolation we used the RNeasy Minikit (Quiagen). 0.5 µg of total mRNA from BA1 cells served as template for reverse transcription with Oligo-dT primers (Invitrogen) and superscript III polymerase (Invitrogen). The q-PCR reaction was performed on a LightCycler II (Roche). Each experiment was done in triplicates and three independent q-PCR reactions were afterwards analyzed with the delta Ct-method. β-actin was used for normalization. Primers are listed in Table 3

	Forward primer	**Reverse primer**
Ezh2 E5-8	GGGCTATCCAGACTGGTGAA	AAATTGCCCACAGTACTCAAGG
Ezh2 E18-19	GTGACCACAGGATAGGCATCT	CAAGGGATTTCCATTTCTCG
Hoxa2	TACGAATTTGAGCGAGAGATTGG	GTCGAGGTCTTGATTGATGAACT
Hoxa3	CCCACAGAAACGCTACACAG	GAGTGGCCCAGAGTTGCTC
Hoxb4	TCCGAGCGCCAGATCAA	CCGAGCGGATCTTGGTGTT
β-actin	CCATCCTGCGTCTGGACCTG	GTAACAGTCCGCCTAGAAGC
Runx2	ATGCTTCATTCGCCTCACAAA	GCACTCACTGACTCGGTTGG
Osterix	TGGCCATGCTGACTGCAGCC	TGGGTAGGCGTCCCCCATGG
ALP	TGACCTTCTCTCCTCCATCC	CTTCCTGGGAGTCTCATCCT
Sox10	CCCACACTACACCGACCAG	GTCGTATATACTGGCTGCTCCC
Sox9	TGAACGCCTTCATGGTGTGG	GTTCTTCACCGACTTCCTCC

Table 3: Primers for q-RT-PCR

4.6 Chromatin immunoprecipitation

For ChIP we followed the protocol described in (Weber, Hellmann et al. 2007). Primers targeting the transcriptional start sites (±1000bp) of the respective genes are listed in Table 4.

	Forward primer	Reverse primer
Hprt	CCAAGACGACCGCATGAGAG	CAACGGAGTGATTGCGCATT
HoxD12	TGGCTCTCAGGTTGGAAAAG	GTCCTCCTATTCCGGGTTGT
Hoxa2	TCTGCTCAAAAGGAGGAGGA	GGAGAAGGCCATGAATTACG
Hoxa3	CCCAGAGGGACTTCAGTCAG	TTACAGCCAGCCTAGGAGGA
Hoxb4	TTCGGAAACAGGAAAACGAG	TGGTCGCTGGGTAGGTAATC

Table 4: Primers for ChIP

4.7 Alcian Blue and Alizarin Red staining

Alcian Blue (Fluka AG) and Alizarin red (Chroma Gesellschaft) stainings were done following the protocols described in (Nagy 2003).

4.8 Microarray

After BA1 cell isolation from E11.5 embryos following the protocol described in chapter 4.3 and mRNA extraction we submitted the samples to Functional Genomics Center Zurich (FGCZ). We used the Affymetrix A430 platform to compare transcriptional differences between *Wnt1-Cre; Ezh2*$^{lox/lox}$ and *Ezh2*$^{lox/lox}$ BA1 cells.

4.9 Statistical analyses

Each experiment was done on at least three independent embryos. Calculations were done in excel, error bars in figures showing either standard error of mean (±SEM) or standard deviation (±SD) as stated in the respective figure legend. Statistical significance was tested with an unpaired, two-tailed Student's t-test.

5 Results

5.1 Inactivation of *Ezh2* in NCCs

First, we had to check whether the conditional knock out model we are using is working in our crossings.

We analyzed targeting the wt-allele, the introduced transgenic construct including the loxP sites or the deletion allele upon Cre-mediated recombination by PCR. This was done on tissue probes of control and Ezh2 cko embryos and mice. The PCR showed the expected bands with 844 bps for the wild-type allele, 591 bps for the floxed allele and 360 bps for the deletion allele (Figure 15).

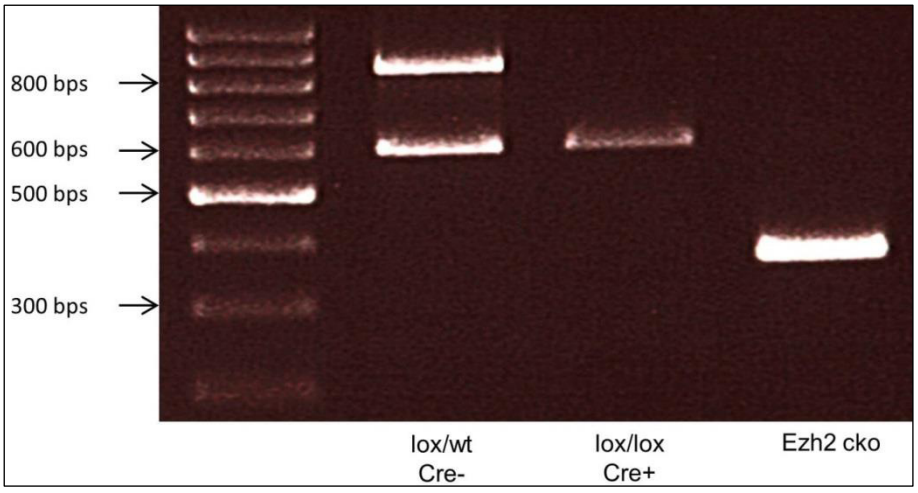

Figure 15: Ezh2-PCR for the different alleles.

$Ezh2^{lox/wt}$;Cre- refers to a Ezh2 wt band of 844 bps and a 591 bps band showing the Ezh2 allele having the loxP sites. The $Ezh2^{lox/lox}$;Cre+ band shows the reduction of genomic DNA when Cre gets expressed in the tissue. And Ezh2 cko refers to a 360 bps long DNA fragment just obtained when Cre and loxP sites are present in the tissue.

To determine the loss of Ezh2 transcripts, we performed quantitative RT-PCR of mRNA isolated from BA1 cells of E11.5 control and Ezh2 cko embryos (Figure 16). We used 2 different primer sets, namely primers targeting Ezh2 Exon 18 and 19 and Ezh2 Exon 5 to 8 (Ezh2 E18-19 and Ezh2 E5-8). The Ezh2 E18-19 primers bind to regions in the mRNA transcript that encodes part of the SET-domain and the Ezh2 E5-8 primer set is targeting a sequence that starts on Exon 5 spanning until Exon 8, therefore lying more 5' to the Ezh2 E18-19 primers. Both quantitative RT-PCRs are showing a comparable and significant reduction of the Ezh2 mRNA transcript in Ezh2 cko embryos when compared to controls. Therefore one can assume that the mRNA transcripts in Ezh2 cko mice are not transcribed or immediately degraded.

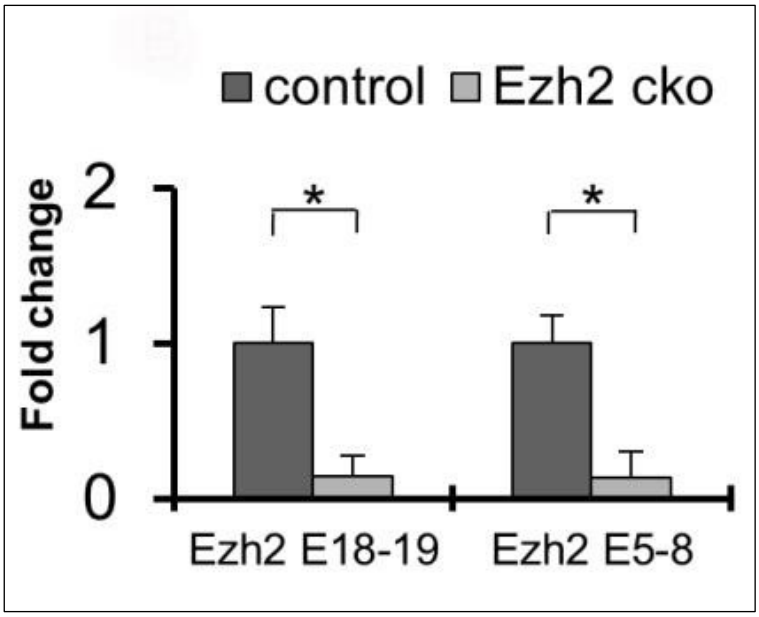

Figure 16: Significant losses of Ezh2 transcripts

A q-RT-PCR with 2 different primer sets revealed a reduction of Ezh2 transcripts in BA1 cells of E11.5 Ezh2 cko embryos when compared to controls. The Ezh2 E18-19 primer targets the SET-domain in the Ezh2 mRNA, the Ezh2 E5-8 primer set binds more 5' than the Ezh2 E18-19 primer set. *P<0.05.

After already seeing that the mRNA levels of Ezh2 are significantly reduced we looked for the major functional read-out of Ezh2 activity and performed immunohistochemistry for H3K27me3 and we observed a loss of the mark in virtually all NCCs that populate the BA1 at E10.5 (Figure 17).

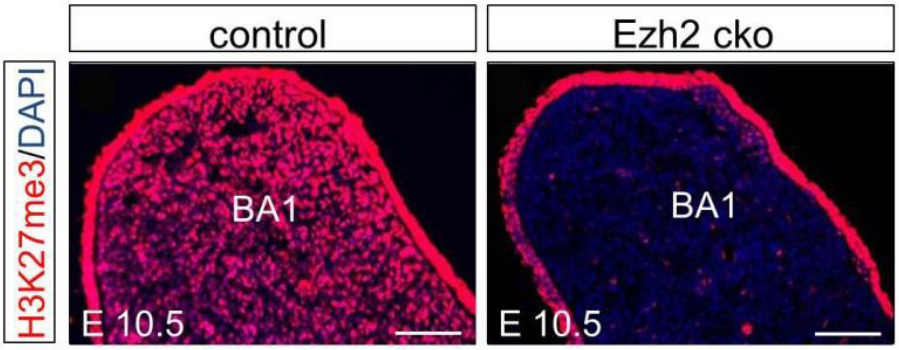

Figure 17: Loss of H3K27me3 in BA1s

Transversal sections of BA1s of control and Ezh2 cko embryos reveals the specific loss of H3K27me3 in the NC derived mesenchyme. The cells of the epidermal layer still have H3K27me3. Scale bars: 50µm.

After being sure that H3K27me3 is gone at early stages of NC development as shown in Figure 17, we examined the persistence of the loss of the H3K27me3 mark at late stages of embryonic development. For that we checked trunk NC derivatives of E17.5 control and Ezh2 cko embryos for H3K27me3 with immunohistochemistry. Both, DRG and AG of Ezh2 cko embryos are still devoid of H3K27 trimethylation at E17.5 upon *Wnt1*-Cre mediated knock-out in NCCs. To mark the structures we double labeled the AG with tyrosine hydroxylase (TH), a terminal differentiation marker for dopaminergic neurons and sensory neurons in the DRG we marked with neurofilament 160 (NF160). Both terminal differentiation markers were present in AG and DRG of control and Ezh2 cko embryos, even though the H3K27me3 mark is gone (Figure 14).

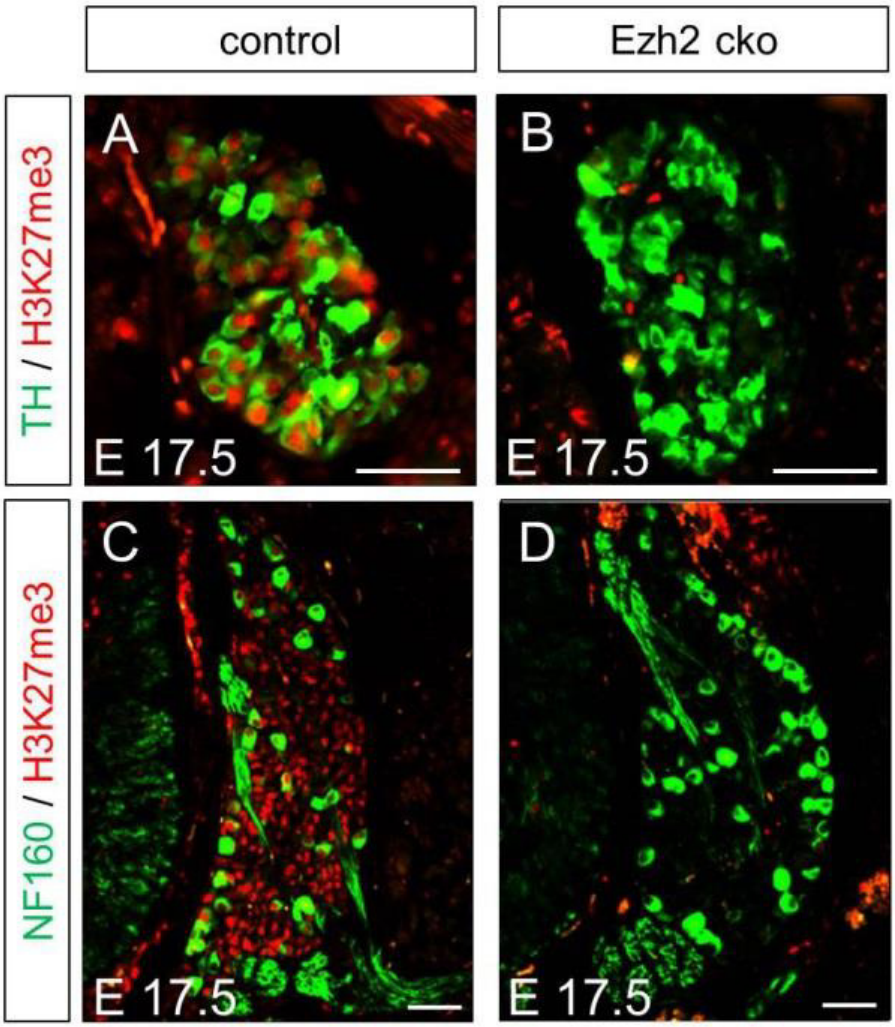

Figure 18: Loss of H3K27 trimethylation in E17.5 Ezh2 cko embryos

Checking for H3K27me3 in trunk neural crest derivatives, like AG and DRG revealed the absence of H3K27 trimethylation in Ezh2 cko mice at E17.5. However, terminal differentiation markers, like TH for dopaminergic neurons and NF160 are still present. Scale bars: 50µm.

Taken together these results demonstrate loss of *Ezh2* activity in NCCs upon a conditional knock out of *Ezh2* in NCCs from early time-points of NC development (E10.5) to late stages like E17.5.

5.2 Migration properties of NCCs are not impaired by loss of *Ezh2*

So we went on and checked for the early event of NC development and the possible effects of conditional *Ezh2* ablation in NCCs. In addition to *Ezh2* ablation, we used the ROSA26 Cre reporter allele (*R26R*) (Soriano 1999), where upon *Wnt1*-Cre mediated recombination all NCCs express β-galactosidase, allowing us to track NCCs that some when in their lifespan expressed Wnt1.

At E10.5 we did not observe differences between Ezh2 cko embryos and controls that were stained with X-Gal for β-galactosidase activity. NCCs migrate to and populate the structures that are supposed to get built up by NCCs, like BAs or DRG (Figure 19).

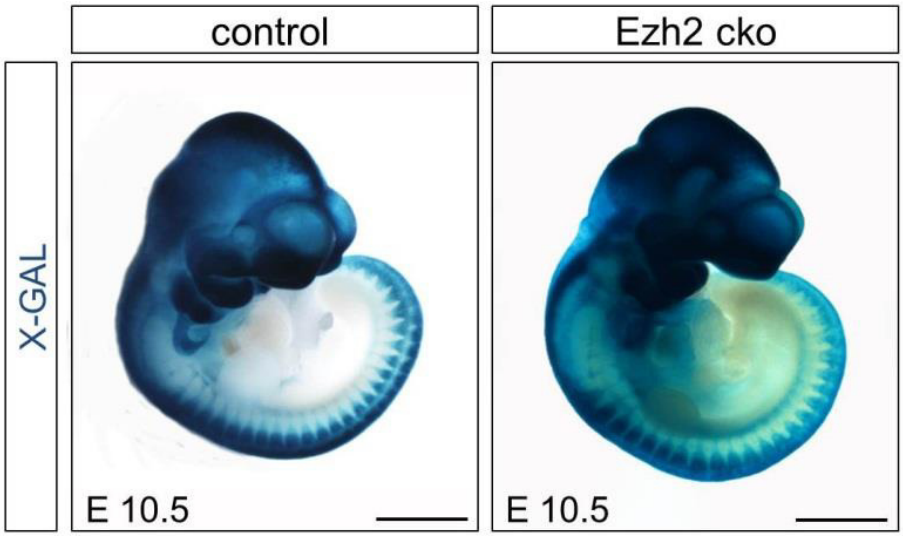

Figure 19: Migration of NCCs is not impaired

The patterns of X-Gal positive cells are the same in E10.5 control and Ezh2 cko embryos, pointing to a normal migration, population and survival in the BAs and trunk derivatives of Ezh2 cko NCCs. Scale bars 2mm.

At E11.5 it is still not possible to distinguish Ezh2 cko embryos from control embryos macroscopically and still the NCCs traced with X-Gal staining show comparable expression patterns (Figure 20).

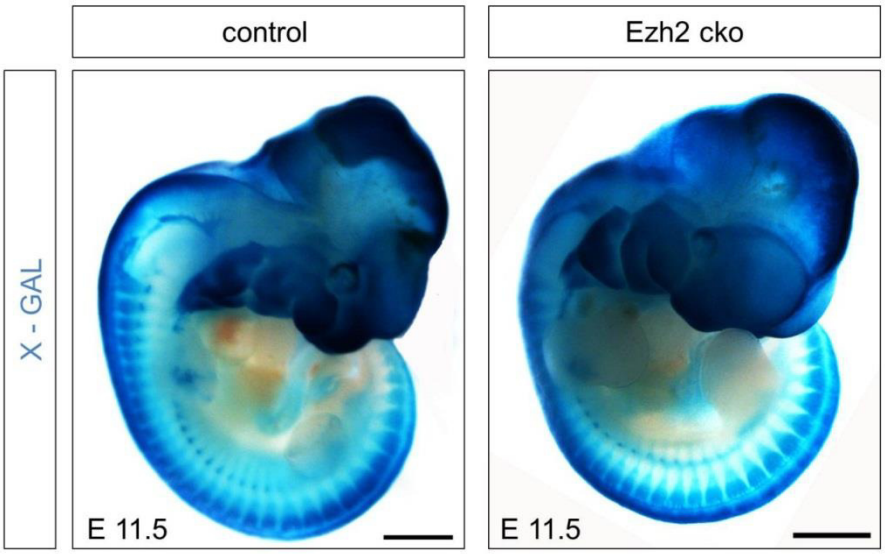

Figure 20: E11.5 Ezh2 cko embryos are still not distinguishable

X-Gal positive cells are still comparable between control and Ezh2 cko embryos at E11.5. Structures are populated and the neural crest cells survive in their environment. Scale bars 2mm.

These results suggest that early events of NC specification, migration and the survival in the respective target structures are not heavily affected by the loss of *Ezh2* in NCCs since up to E11.5 control and Ezh2 cko embryos are not distinguishable from each other when staining with X-Gal for NCCs.

5.3 *Ezh2* depletion in NCCs causes severe craniofacial defects

After realizing that the early events of NC development, like specification, migration and population of target structures is not impaired upon conditional deletion of *Ezh2* in NCCs we decided to look at later stages of development. At E12.5 we were able to distinguish between Ezh2 cko and control embryos since the phenotype becomes apparent (Figure 21). At this stage the remodeling process of the intermediate BAs and FNP NCCs starts and hey build up their final structures. The Ezh2 cko embryos lack the snout but are still alive.

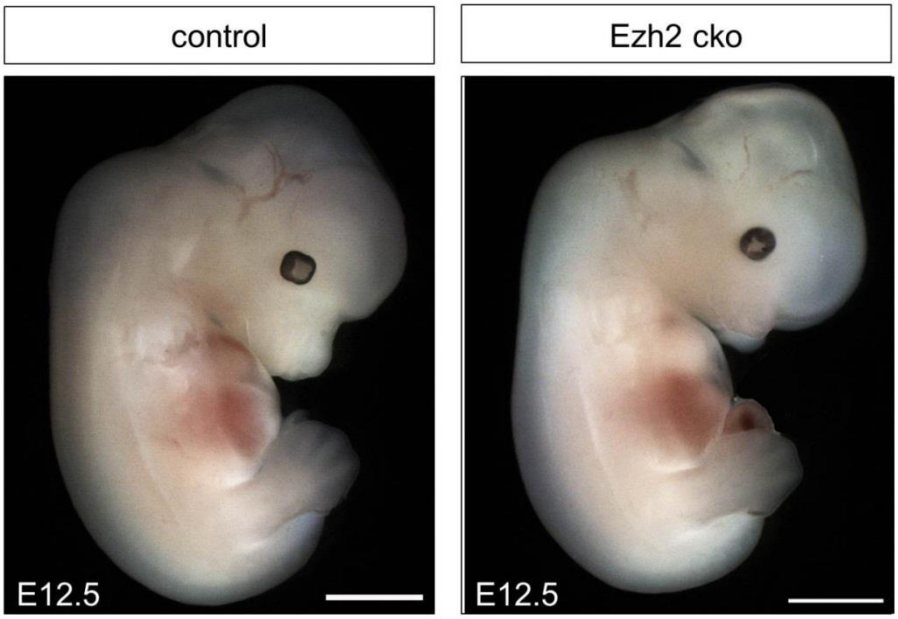

Figure 21: The phenotype becomes apparent at E12.5

In Ezh2 cko embryos the phenotype becomes appearent at E12.5. Most remarkebly, they lack the snout pointing to a loss of craniofacial derivatives, derived from cranial neural crest cells. Contrary to the facial region, the body looks normal. Scale bars: 2mm.

The phenotype is shown on E14.5 Ezh2 cko embryos carrying the *R26R* allele and stained for X-Gal (Figure 22). Ezh2 cko embryos are missing the complete craniofacial region, although some residual cells are still positive for β-galactosidase

activity (Figure 12). The Ezh2 cko embryos change their cranial morphology due to the absence of the stabilizing tissue of the craniofacial region and therefore the head bends over. In addition we observed that Ezh2 cko embryos with the *R26R* allele have peripheral nerves (arrowheads) at the level of the hindlimbs and these are comparable to their control littermates what points to a normal development of peripheral nerves in Ezh2 cko embryos (Figure 22).

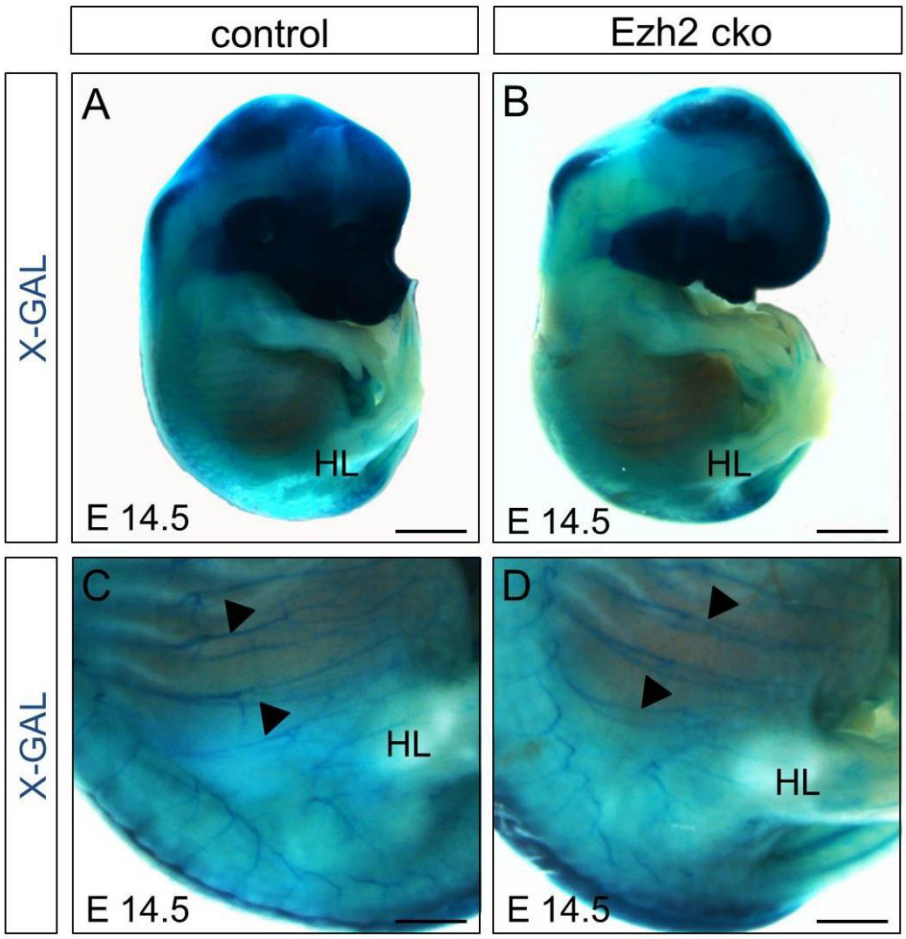

Figure 22: Phenotype on X-Gal stained E14.5 embryos

A and B: E14.5 embryos stained with X-Gal show the craniofacial phenotype already observed at E12.5. But even in the head region residual X-Gal positive cells are seen.

C and D: higher magnification of the hindlimb region of the embryos shown in A and B. The arrowheads point to peripheral nerve that seem unaffected.

Scale bars: A and B: 2mm; C and D: 1mm; HL: hindlimbs

The embryos survive to late developmental stages but never got born. The latest studies we performed on embryos were at E17.5. At this stage the embryos show comparable size in total but the missing structures from the craniofacial areas cause a severe phenotype in the head region (Figure 23).

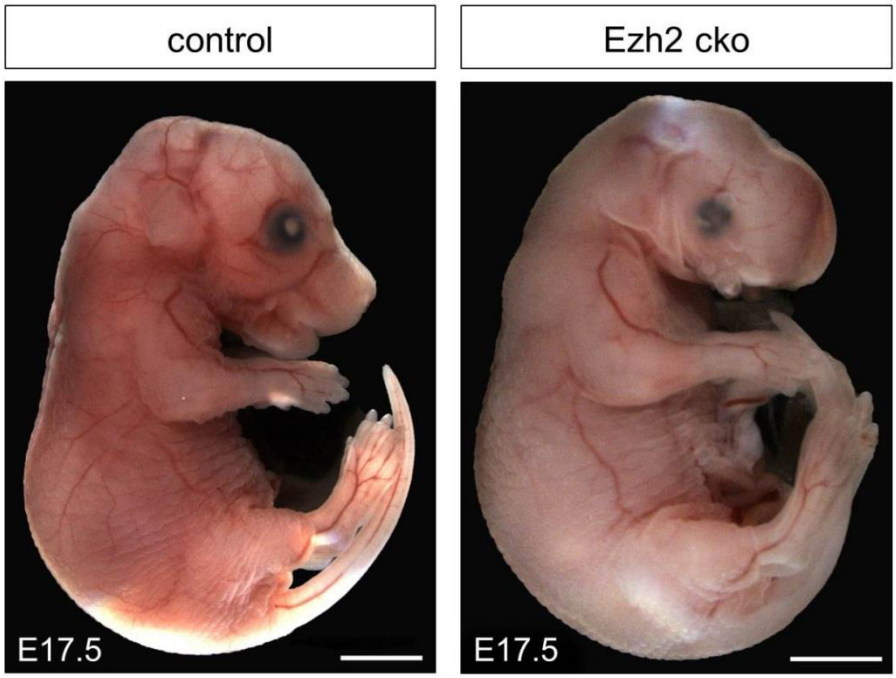

Figure 23: Severe craniofacial phenotype upon Ezh2 cko at E17.5

The phenotype shown on E17.5 embryos. Looking quite normal in trunk regions, the massive phenotype, the lack of craniofacial structures, prevents viability since we never saw newborns. Scale bars: 2mm.

5.4 Ezh2 is not required for differentiation of trunk NCCs

To check for the role of Ezh2 in trunk NCC differentiation we performed immunohistochemistry stainings on E17.5 embryos of Ezh2 cko and control embryos transversal sections. At these later stages of development any phenotype might have manifested and would be easily detectable.

First checking for sensory neurogenesis we stained for Brn3a, a marker for progenitor cells of the sensory lineage in DRG (Eng, Lanier et al. 2004). Additionally we stained the DRG for NF160 to check for terminal differentiation of neurons. Both markers showed no obvious differences when comparing control to Ezh2 cko DRG (A and B in Figure24).

Then we did stainings for the progenitor and terminal differentiation markers of neuronal, autonomous differentiation. Mash1, a progenitor marker for autonomous neurons is not changed. To visualize the AG we stained for NeurofilamentM in addition to Mash1 (C and D in Figure24). Further checking for tyrosine hydroxylase (TH), a marker for terminal differentiated autonomic neurons and again neurofilamentM showing the presence of both in AG when comparing E17.5 Ezh2 cko embryos with control embryos (E and F in Figure24).

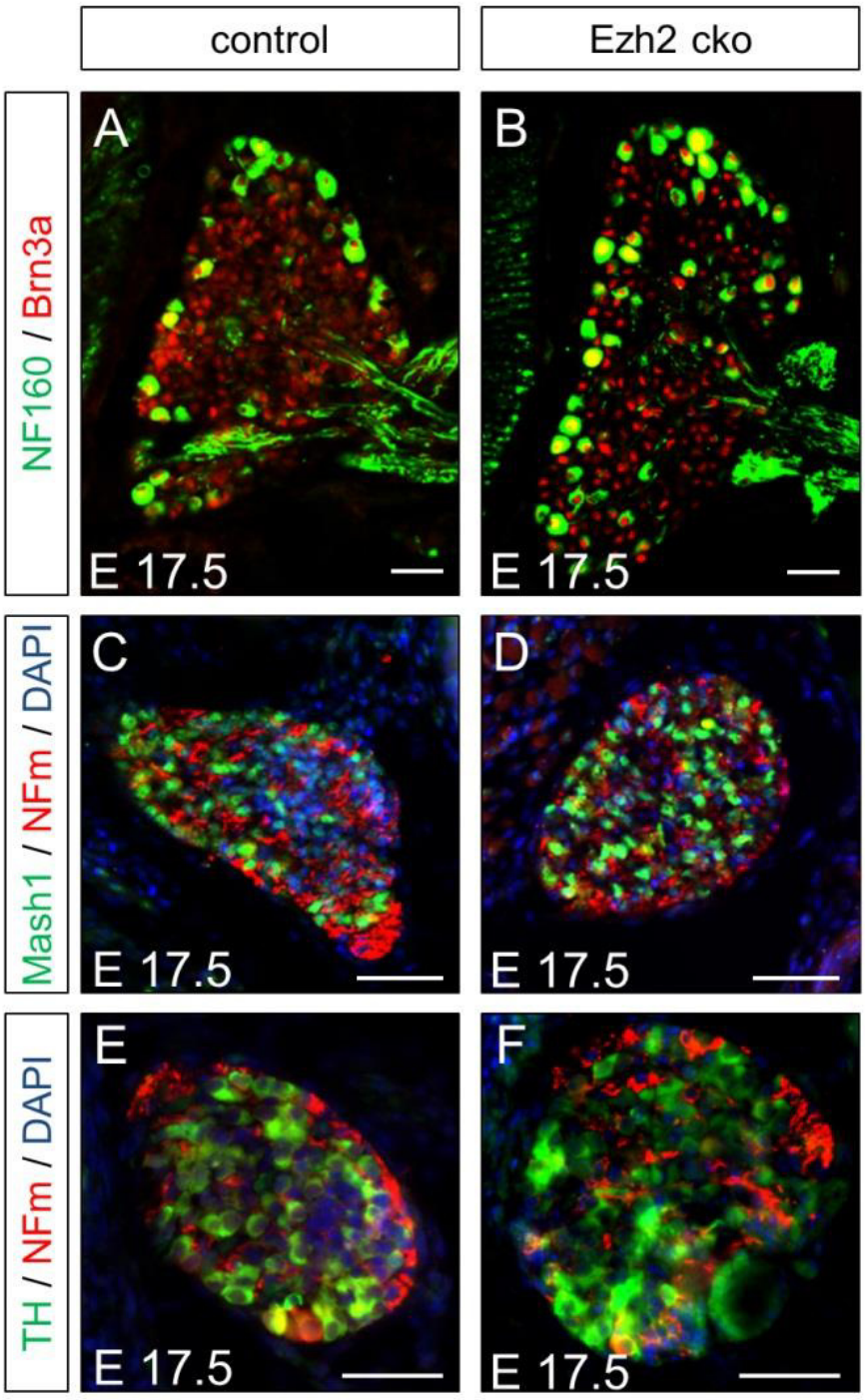

Figure24: Immunohistochemistry for neuronal markers of trunk NCCs

A and B showing DRG of control and Ezh2 cko embryos stained for Brn3a a sensory, neuronal progenitor marker and NF160 to mark terminal differentiated neurons, showing no differences.

C – F showing AG of control and Ezh2 cko E17.5 embryos with no obvious differences in progenitor marker expression (Mash1 in C,D) and terminal dopaminergic marker expression (TH in E,F). Double staining with NFm marks the location of the AG.

Scale bars: 50µm

After excluding possible neuronal differentiation defects we thought about the glial lineage of trunk NCCs. So we looked for Schwann cells, the myelin producing cells of the peripheral nervous system, checking for a marker expressed in premature Schwann cells and we were able to find cells expressing Oct-6 in Ezh2 cko mice comparable to controls peripheral nerves. To mark the peripheral nerves we stained for NF160 additionally to Oct-6 (E and F in Figure 25).

Next we wanted to see whether glial differentiation markers in DRG are changed and we were not able to examine differences comparing Ezh2 cko embryos against control embryos. We performed immunohistochemistry for glial fibrillary acidic protein (GFAP) a late marker of glial differentiation. At E17.5 we couldn't see changes in the expression of GFAP in DRG when comparing Ezh2 cko embryos against control littermates (A and B in Figure 25). Further we performed immunohistochemistry for an early marker of glial differentiation, like fatty acid binding protein (FABP). This marker didn't show alterations upon Ezh2 ablation, too (C and D in Figure 25). These results lead us to the conclusion that we are not interfering in the differentiation steps of trunk NCCs in DRGs and AGs neither neuronal nor glial in these later developmental stages.

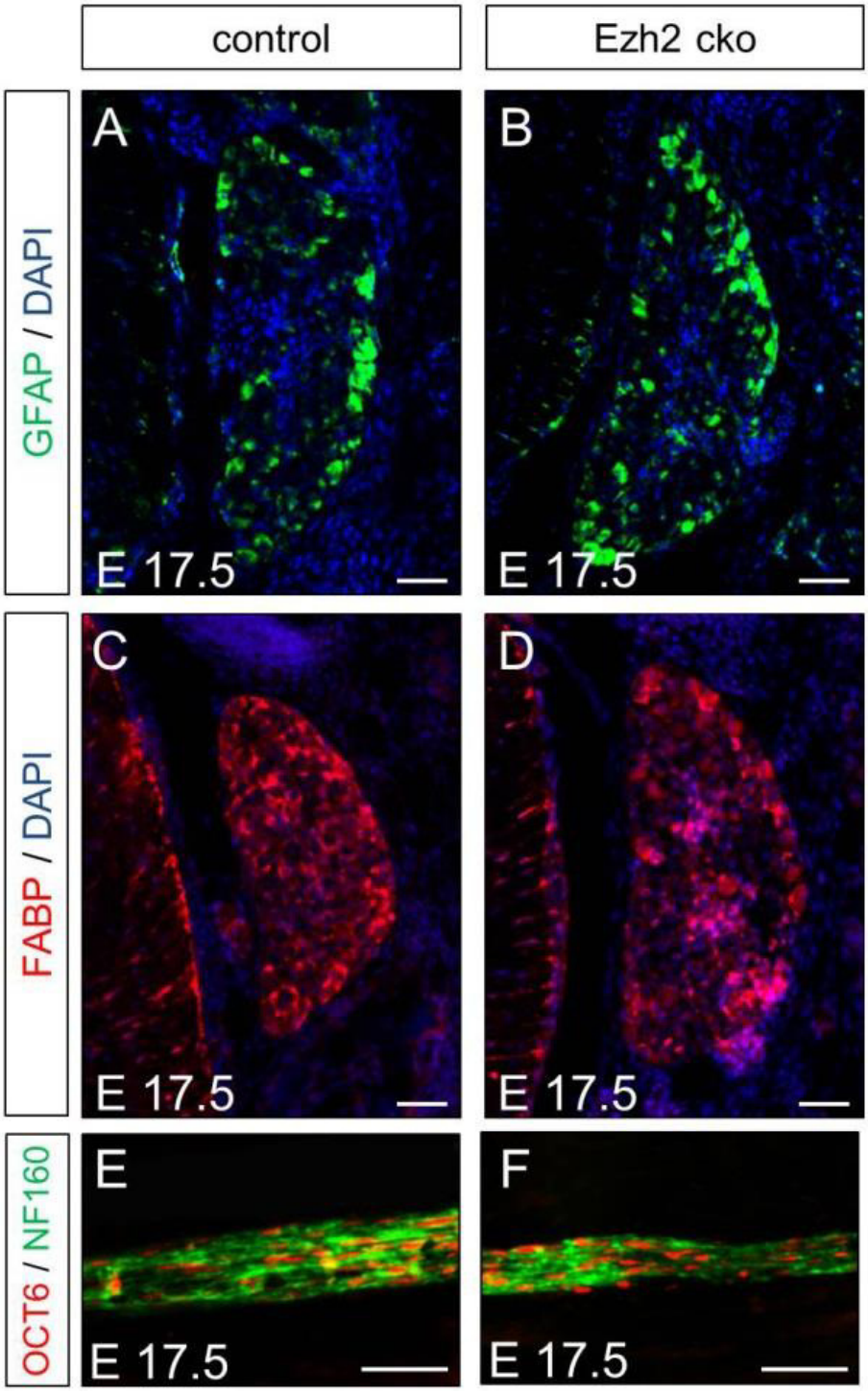

Figure 25: Immunohistochemistry for glial markers of trunk NCCs

A and B showing presence of GFAP a late glial marker in DRG of control and Ezh2 cko animals.

C and D showing no differences in FABP abundance, an early glial marker.

E and F showing peripheral nerves, marked with NF160 of Ezh2 cko and control animals and both have the cmparable Oct6 expression a marker for premature Schwann cells.

Scale bars: 50µm.

5.5 Ezh2 cko embryos lack chondrogenic and skeletal elements

The conditional ablation of *Ezh2* in NCCs results in the loss of all chondrogenic structures that build up the skeletogenic elements of the craniofacial structures as shown by alcian blue staining. At E14.5 Ezh2 cko embryos are lacking the upper and lower jaw and the nasofrontal plate (Figure 26). All these derivatives are built up by CNCCs since this subpopulation of NCCs has mesenchymal potential (see chapter 3.1.4.1). Partially, these chondrogenic elements are just a template for bones therefore we checked for later stages, too.

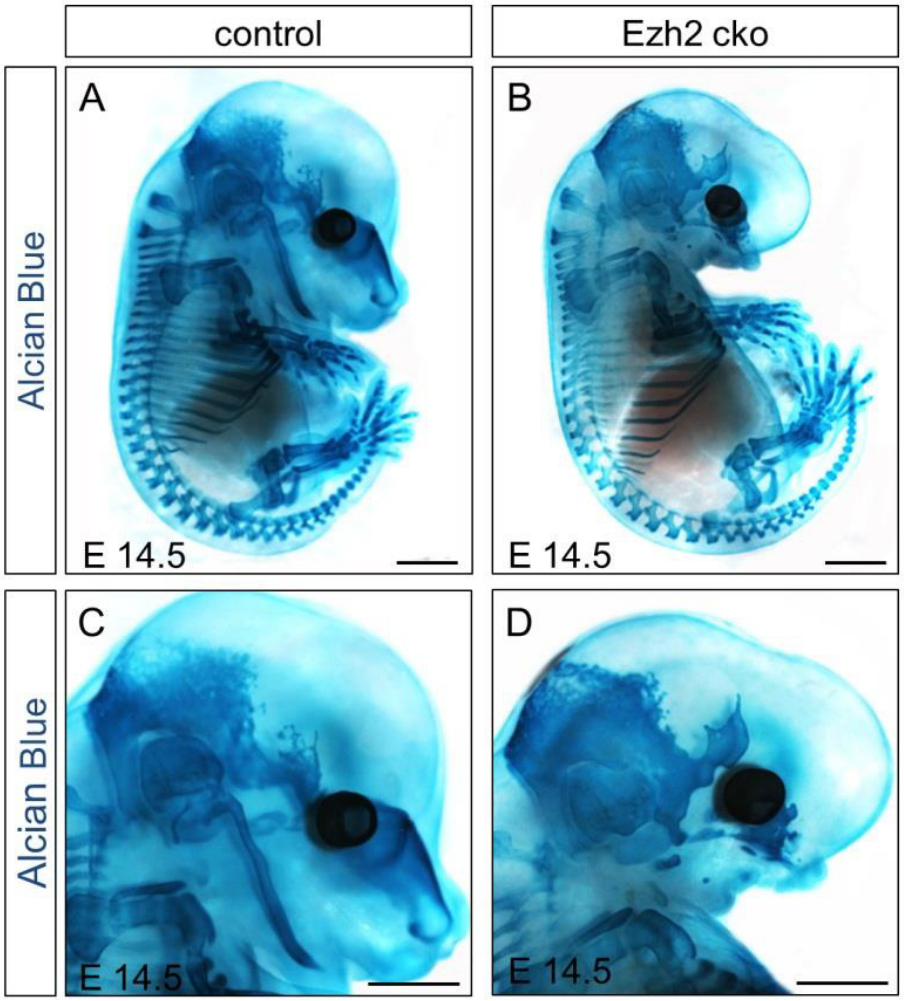

Figure 26: E14.5 Ezh2 cko embryos show a loss of chondrogenic elements in the facial region

A and B: The loss of chondrogenic elements in the facial region of E14.5 Ezh2 cko is shown by Alcian Blue staining.

C and D show higher magnification of the head region of the embryos in A and B. Scale bars 2mm.

At E17.5 almost all facial skeletal elements are absent in Ezh2 cko embryos as shown in Figure 23 and Figure 27. Elements completely derived from CNCCs are missing, whereas skull bones in which CNCCs are just contributing to, look abnormal but are established (Jiang, Iseki et al. 2002; Gross and Hanken 2008). We still see parts of the parietal skeletal plate (p in Figure 27) and the interparietal plate exists (ip in Figure 27). Bones derived exclusively from CNCCs, like the mandible, maxilliary (md and mx in Figure 27) or the frontal-plate (f in Figure 27) is completely missing in Ezh2 cko embryos at E17.5. Chondrogenic elements are partially established as seen from the otic capsule (o in Figure 27) but more rostrally/anterior the chondrogenic elements look abnormal.

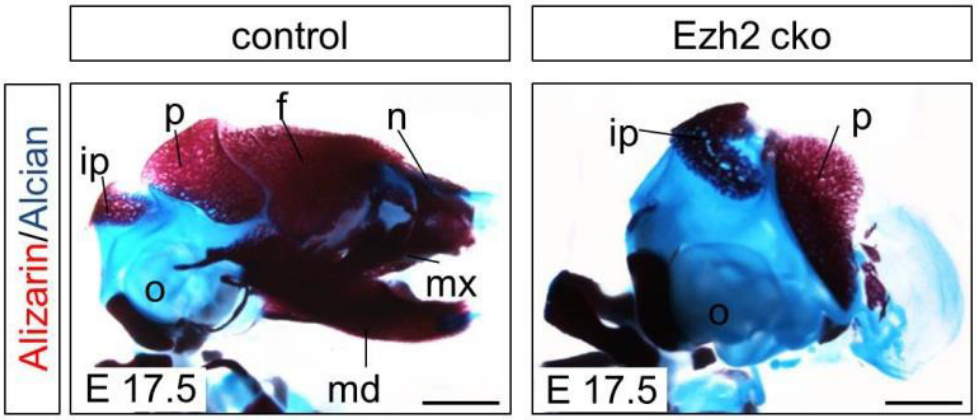

Figure 27: Almost all neural crest derived craniofacial elements are missing in E17.5 Ezh2 cko embryos

A combined Alizarin Red and Alcian blue staining on E17.5 control and Ezh2 cko embryos shows a loss of chondrogenic and skeletogenic elements in the facial region of Ezh2 cko embryos when compared to control littermates.

Mx: maxilliary; md: mandible; n: nasal plate; f: frontal plate; p: parietal plate; ip: interparietal plate; o: otic capsule. Scale bars 2mm.

To elucidate the observed phenotype further that becomes apparent at E12.5 and causes a massive loss of craniofacial NC derivatives at later stages, we decided to check for the precursor cells that build up these structures.

5.6 The transition of NCSCs to MPCs

After the observation that chondrogenic and skeletogenic elements are missing from E12.5 onwards in Ezh2 cko embryos, we asked about the possible cause of this phenotype. So we decided to check for cells that differentiate into these derivatives. To differentiate into the mesenchymal derivatives of the NC, NCSCs migrating from hindbrain levels, caudal to rhombomere 8, undergo a Tgf-β mediated transition to become MPCs. These MPCs populate the BAs and subsequently differentiate into chondro- and skeletogenic elements of the facial area (see chapter 3.1.4.1). This transition step is accompanied by the downregulation of the NCSC transcription factor Sox10 and upregulation of the MPC marker Sox9. In order to understand whether this transition is affected by Ezh2 conditional ablation we performed a q-RT-PCR on isolated BA1 cells for Sox10 and Sox9 (Figure 28). No statistically significant differences were seen between BA1 cells of Ezh2 cko embryos compared to control littermates.

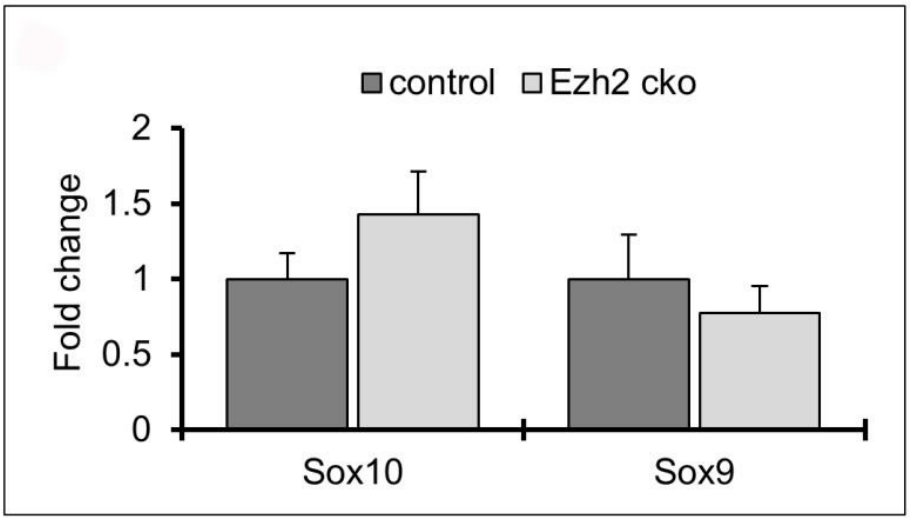

Figure 28: Sox10 and Sox9 transcript levels are not changed

Slight but not significant changes in Sox10 and Sox9 transcripts when comparing controls to Ezh2 cko BA1 cells with q-RT-PCR. ±SEM.

5.7 Cell cycle properties of mesenchymal progenitor cells

One defining criteria for stem/progenitor cells is their self-renewal capacity (chapter 3.1.2). So we decided to check for the cell cycle properties of MPCs in Ezh2 cko mice. A prominent marker for cells in the cell cycle is Ki67. The protein is expressed in all phases of the cell cycle, just not in cells that are in G0. When staining for Ki67 we were not able to observe any major differences in Ki67 abundance in BA1 cells of Ezh2 cko and control BA1's (Figure 29).

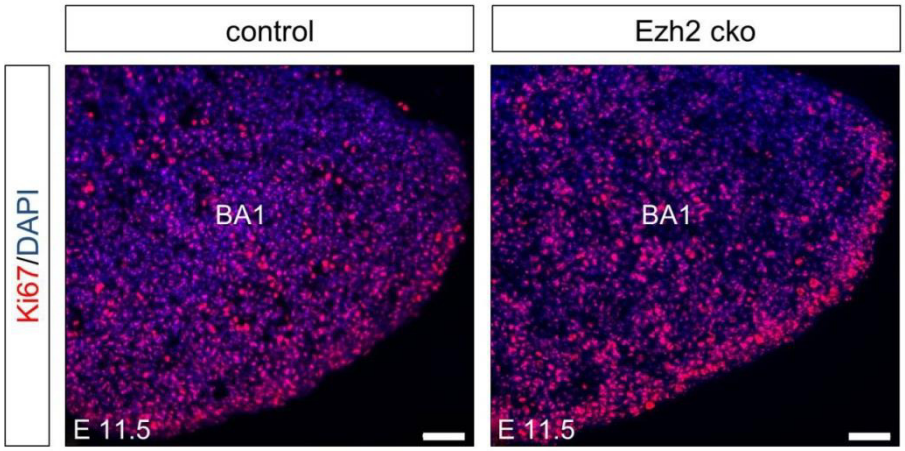

Figure 29: Ki67 expression is not changed

The cell cycle marker Ki67 is not changed in Ezh2 cko BA1's when compared to control BA1's with immunohistochemistry at E11.5. Scale bars 50µm.

Since Ki67 is just giving an indication of cells that are currently in the cell cycle we performed further experiments to check for specific phases of the cell cycle. Next we examined cells undergoing S-Phase via a pulse of EdU 1 hour prior to euthanization of the animals at E11.5 (Figure 30). The quantification showed no significant differences in the number of cells that are positive for EdU in the BA1 when comparing controls to Ezh2 cko mice. For control animals we counted 46,6 % of EdU positive cells and for Ezh2 cko 47,8 % EdU positive cells, respectively.

Therefore we concluded that cells entering and exiting the S-Phase during a 1 hour period of EdU incorporation are not affected.

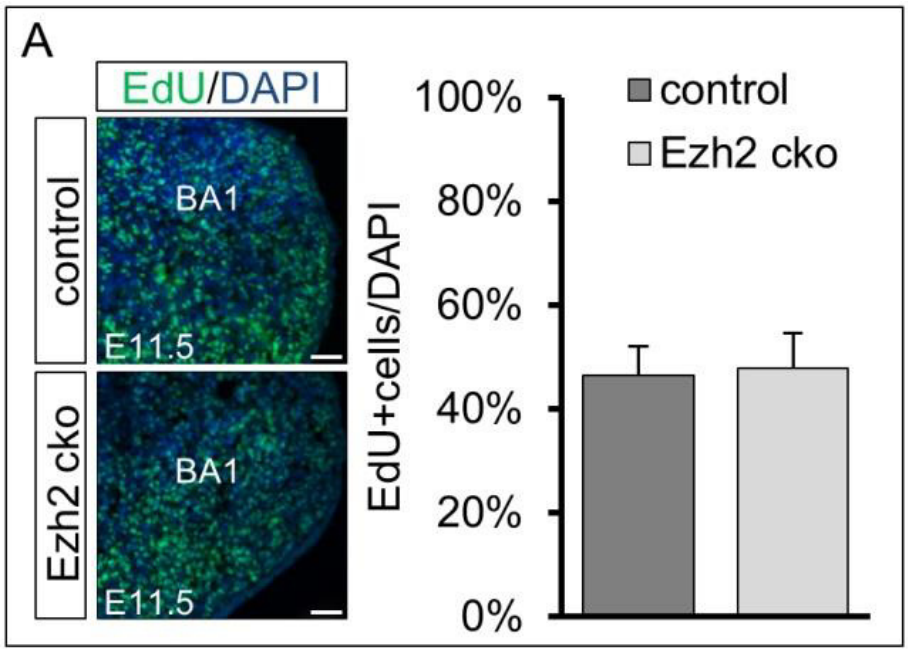

Figure 30: Comparison of S-phase cells

The immunohistochemical stainings for the thymidin analogon EdU are depicting the cells in BA1 that are in or went through S-phase within a 1 hour time frame of control and Ezh2 cko embryos 1 hour after injection and sacrification of the animals. The quantification didn't reveal a significant change in the number of EdU positive, due to a loss of Ezh2 in NCCs. ±SEM

Next we checked for cells that are in M-Phase performing a PHH3 staining on BA1 sections of control and Ezh2 cko mice at E11.5 (Figure 31). Again no significant differences could be found between control and Ezh2 cko animals. BA1 sections of control animals contained 3,9 % phospho-histone positive and the BA1 of Ezh2 cko animals yielded 5,1 % of PHH3 positive cells.

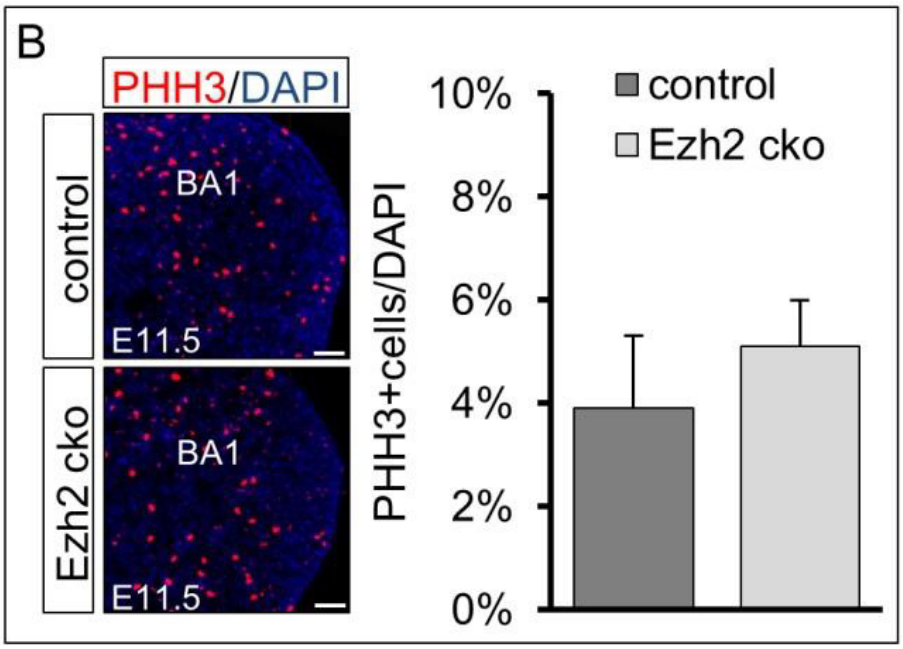

Figure 31: Comparison of PHH3 positive cells in BA1's

The quantification shows the percentual proportions of PHH3 positive cells in BA1 (DAPI positive cells) of control and Ezh2 cko embryos. ±SEM

Lastly, to definitely exclude any cell cycle difference we performed cell cycle fluorescence activated cell sorting (FACS) on isolated E11.5 BA1 cells of control and Ezh2 cko embryos. The cell cycle profiles of control and Ezh2 cko BA1 cells are shown in Figure 32, showing comparable amounts of cells are in the respective phases of the cell cycle when comparing BA1 cells of E11.5 control and Ezh2 cko embryos.

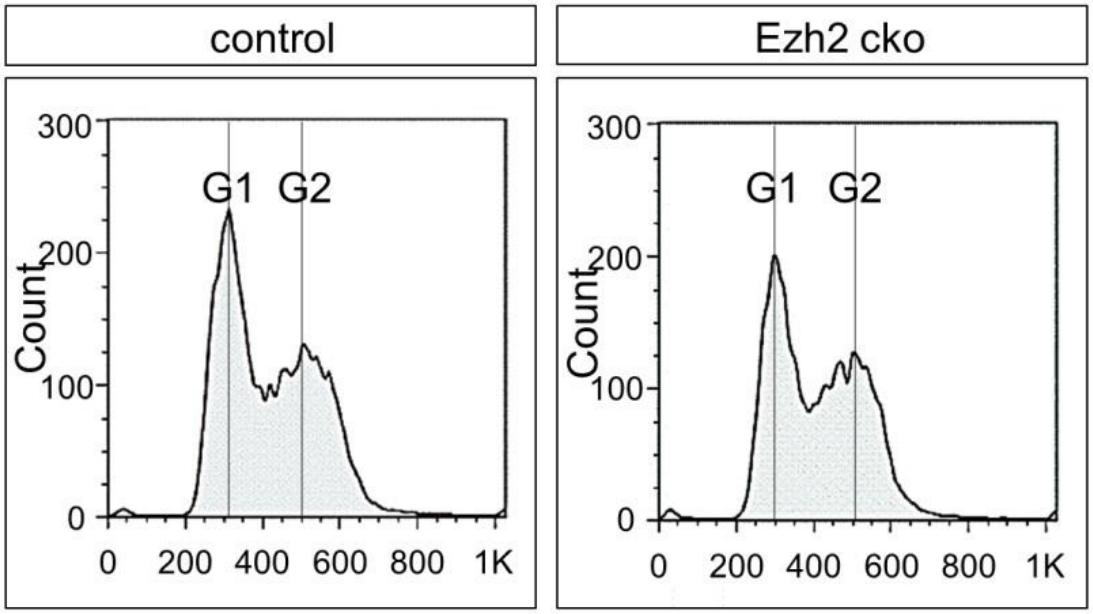

Figure 32: Cell cycle FACS profiles of E11.5 BA1 cells

The cell cycle FACS profiles of BA1 cells from E11.5 control and Ezh2 cko littermates didn't reveal changes in different phases of the cell cycle, like G1 and G2.

The quantification of the cell cycle FACS profiles (Figure 32) was done with the Dean-Jett-Fox algorithm provided by FlowJo, published in (Fox 1980). The algorithm calculated comparable and not significant proportions of cell cycle phases for control and Ezh2 cko embryos BA1 cells at E11.5. We find around 31% of the cells in G2-phase and 38% are detected to be in G1-phase of the cell cycle for both genotypes. Additionally, the algorhithm provides a mathematical model to estimate the cells that are currently in S-phase. The proportion of cells that are currently in S-phase, is approximately 30% in both cases.

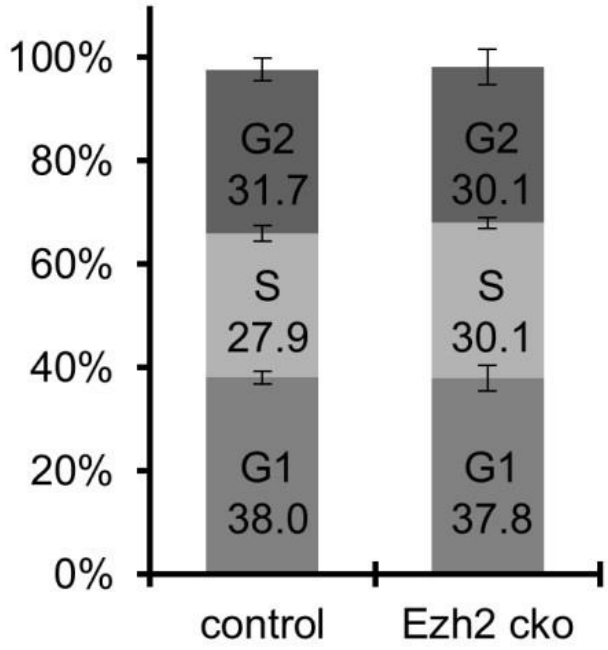

Figure 33: Proportions of BA1 cells in different cell cycle phases

The quantification of the cell cycle FACS doesn't show changes in the proportions of BA1 cells in each phasewhen comparing control to Ezh2 cko BA1 cells. The Dean-Jett-Fox algorhythm provides a method to estimate cells that are currently in S-phase in a cell cycle FACS. ±SEM

After analyzing the cell cycle properties of BA1 cells, we checked for apoptosis. Apoptosis could be another event that BA1 cells suffer from and with an impaired survival of MPCs Ezh2 cko embryos woud lack craniofacial elements.But after staining for Caspase-3 a marker for cells inevitably gone into apotosis we did not see differences in the rates of Caspase-3 positive cells between Ezh2 cko embryos and controls at E11.5 (Figure 34).

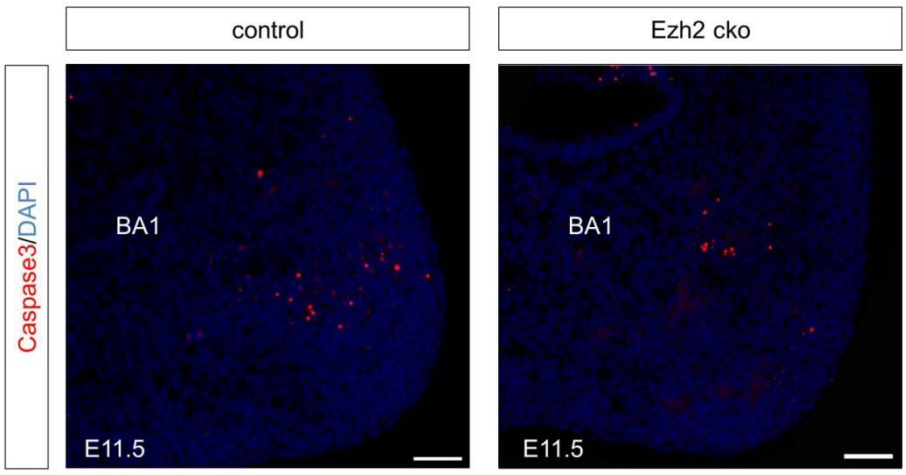

Figure 34: No increased apoptosis rates in Ezh2 cko embryos at E11.5

A staining for Caspase3, to mark cells that irreversibel entered apoptosis, didn't show differences when comparing BA1's of E11.5 control and Ezh2 cko littermates. Scale bars: 50µm.

Overall these results demonstrate that Ezh2 conditional knock out in NCCs doesn't change, in the first step the transition of NCSCs to MPCs and subsequently the cell cycle properties of MPCs that populate the BAs suggesting that our phenotype is not due to cell cycle mis-regulation or apoptosis. That apoptotic rates don't increase might be due to the fact that we are interfering with domains of cells in BAs. So we decided to check for the next step, the differentiation of BA1 cells into mesenchymal derivatives. BA1 cells undergo chondrogenic and skeletogenic differentiation to build up the bones of the craniofacial region.

5.8 First indications of chondro-/osteogenic differentiation impairment

After checking many properties of the MPCs transition stage and seeing that the MPC by itself is not severely affected and can be established from NCSCs (Figure 28) and has normal cell cycle properties (Figure 29, Figure 30, Figure 31, Figure 32, Figure 33) we asked about the next progenitor step in the mesenchymal differentiation process. So we checked for early markers of osteogenic and chondrogenic differentiation in BA1 cells. The first indication for an impairment of chondrogenic/skeletogenic differentiation is the absence of Collagen2a1 (Col2a1) expression in the BA1 at E11.5 in Ezh2 cko embryos that was not observed in control littermates (Figure 35).

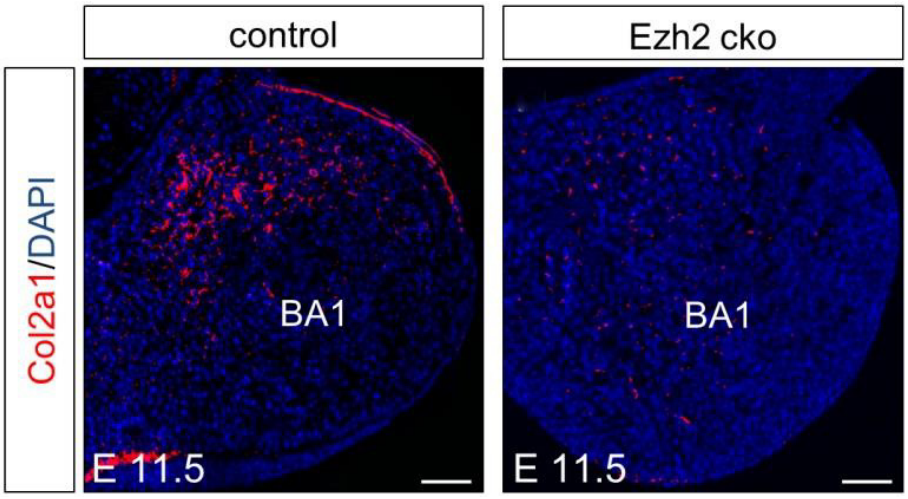

Figure 35: Impairment of chondrogenic differentiation at E11.5

Col2a1 a marker for chondrogenesis is absent in E11.5 Ezh2 cko BA1's pointing to a chondrogenic differentiation impairment. Scale bars 50µm.

After finding an indication for chondrogenic differentiation impairment, we further found that Runx2, Osterix and alkaline phosphatase (ALP) are significantly down regulated in Ezh2 cko BA1 cells when compared to control BA1 cells as shown by quantitative RT-PCR (Figure 36). Runx2, Osterix and ALP are known markers for osteogenic differentiation (Nishimura, Hata et al. 2012). Whereas Runx2 is considered to be the main transcription factor for osteogenic differentiation and directly regulates Osterix is ALP a later marker for osteogenic differentiation.

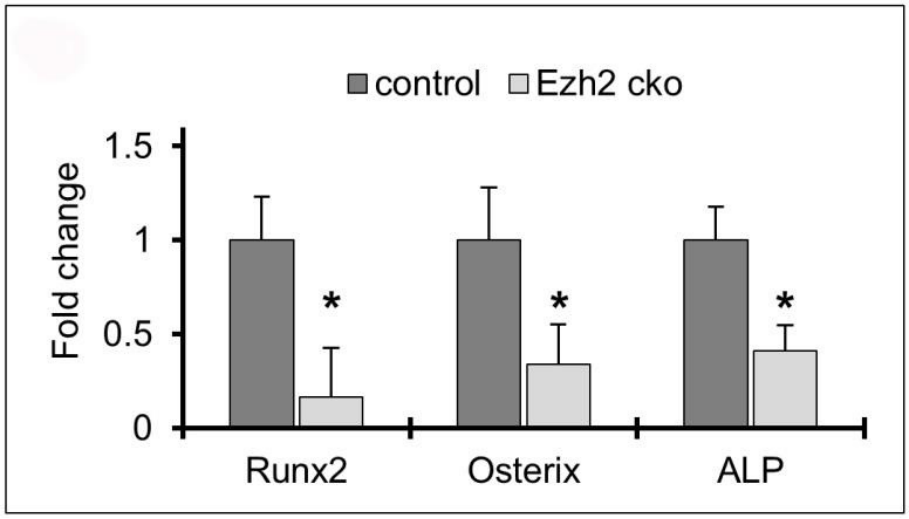

Figure 36: Osteogenic markers are downregulated

Q-RT-PCR revealed the significant downregulation of osteogenic markers in BA1 cells of E11.5 Ezh2 cko embryos. Runx2 as key gene for osteogenic regulation, affects directly Osertix expression. Alkaline phosphatase is a later osteogenic marker. *P<0.05.

Together these results show that the impairment of CNCCs to build up the chondro-skeletogenic elements starts earlier than the actual differentiation of the cells. The examinations of BA1 cells of control and Ezh2 cko embryos at E11.5 already shows the lack of early differentiation markers and effectors of the chondrogenic and skeletogenic lineages.

5.9 Transcriptome analysis on BA1 cells

After checking the transition of MPCs to osteoblasts and chondrogenic progenitors and finding first indications of chondro-/skeletogenic differentiation impairment, we decided to perform a microarray on BA1 cells to determine putative candidate genes that drive the phenotype in Ezh2 cko embryos.

We performed an array on the Affymetrix A430 platform with 3 independent replicates for each genotype. The clustering of the replicates was consistent and comparable among the genotypes as shown by the heatmap of genes that are at least 2-fold up- or downregulated with a p-value <0.01 (Figure 37).

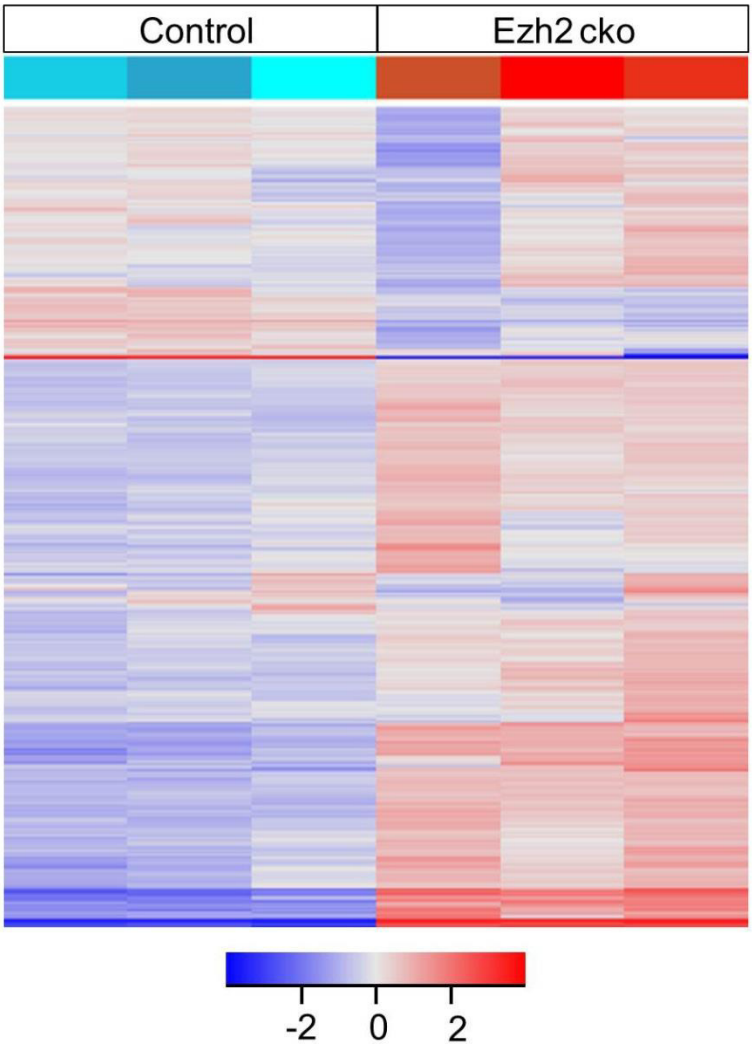

Figure 37: Heatmap of microarray

The microarray, using the Affymetrix A430 system showed a clustering of the genotypes. 3 independent samples of each genotype (*Ezh2*$^{f/f}$ and *Ezh2*$^{f/f}$*;Wnt1-Cre*) were used.

Z-Value is given color coded below the map.

Since Ezh2 mainly acts mainly as a repressor we first focused on the upregulated transcripts in Ezh2 cko BA1 cells with newly set parameters. Strikingly, many Hox gene transcripts were increased in Ezh2 cko BA1 cells, when we decreased the p-value ($p<0.001$) to get highly significant differences as shown in Table 5.

Gene symbol	ratio	p-value
Hoxa9	134.3	1.43E-09
Hoxa10	104.2	1.79E-11
Hoxc8	56.2	3.12E-09
Hoxb3	21.73	3.11E-08
Hoxd13	21.47	1.01E-08
Hoxc10	15.36	7.08E-06
Hoxc4	12.12	4.17E-08
Hoxa11	10.53	2.14E-06
Hoxb13	10.2	4.03E-07
Hoxa2	8.504	3.14E-08
Hoxa5	4.95	0.0001511
Hoxc9	4.338	1.78E-08
Hoxa3	4.097	0.0001228
Hoxc5	3.466	6.58E-06
Hoxc13	2.592	2.73E-05
Hoxd8	2.501	4.62E-05
Hoxa1	2.355	1.21E-06
Hoxb2	2.122	2.22E-05
Hoxa7	2.089	6.43E-06
Hoxb4	2.013	0.0001119

Table 5: Hox gene overexpression in Ezh2 cko BA1 cells

The micorarray showed a highly significant upregulation of many Hox genes. Normally, neural crest cells from the BA1 should be devoid of *Hox* gene expression. In Ezh2 cko BA1 cells almost all Hox genes get significantly upregulated when compared to control BA1 cells. The Hox genes underlayed with a green bar are known blockers of skeletogenesis.

The overexpression of *Hox* genes points again to the already described function of the PcG complexes to regulate *Hox* gene expression during embryonic development. Further the *Hox* genes are known repressors of skeletogenic differentiation in chicken (Couly, Grapin-Botton et al. 1998; Creuzet, Couly et al. 2002), so we decided to focus on the 3 *Hox* genes described in these papers. The 3 candidates are marked with the light green bar in Table 5, namely Hoxa2, Hoxa3 and Hoxb4*,* with overexpression levels of 8.50-, 4.10- and 2.01-fold, respectively. All three genes were highly significant upregulated in the microarray when comparing BA1 cells from Ezh2 cko embryos with BA1 cells from control littermates (Table 5). To validate this we performed quantitative RT-PCR confirming the highly significant upregulation ($p<0.001$) of the 3 *Hox* genes that we selected. Hoxa2 was upregulated 16.48 fold, for Hoxa3 there was an upregulation of 2.58 times observed and Hoxb4 had a value of 6.51 times up when comparing Ezh2 cko BA1 to control BA1 cells in the quantitative RT-PCR (Figure 38).

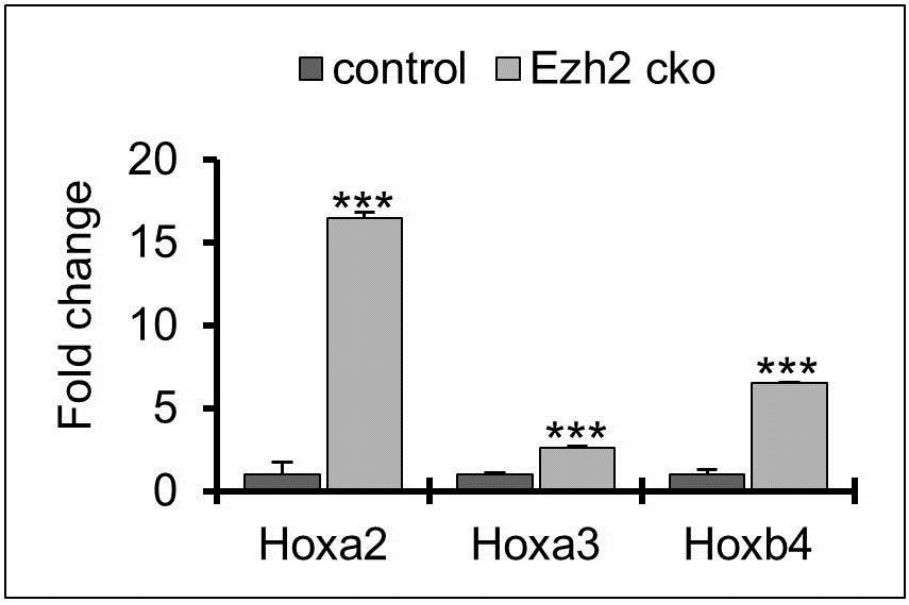

Figure 38: Hoxa2, Hoxa3 and Hoxb4 overexpression

q-RT-PCR confirmed the highly significant upregulation of Hoxa2, Hoxa3 and Hoxb4 in BA1 cells of Ezh2 cko embryos when compared to control embryos. ***P<0.001; ±S.E.M.

5.10 H3K27me3 occupancy on *Hoxa2*, *Hoxa3* and *Hoxb4* transcriptional start sites in BA1 cells

To exclude the possibility that Hoxa2, Hoxa3 and Hoxb4 get upregulated due to a secondary effect and are not a direct target of H3K27me3 in BA1 cells, we performed chromatin immunoprecipitation (ChIP) for H3K27me3 and checked the abundance of DNA with primers targeting the transcriptional start sites (TSS) of Hoxa2, Hoxa3 and Hoxb4 upon enrichment of chromatin with an antibody against H3K27me3. To control our results we used a positive control, Hoxd12 that should not be expressed in BA1 cells and compared the abundance of target gene DNA against abundance of a negative control, namely Hypoxanthine–Guanine Phosphoribosyltransferase (HPRT) a housekeeping gene that should be devoid of H3K27 trimethylation, since the H3K27me3 mark is found on silent gene promotors.

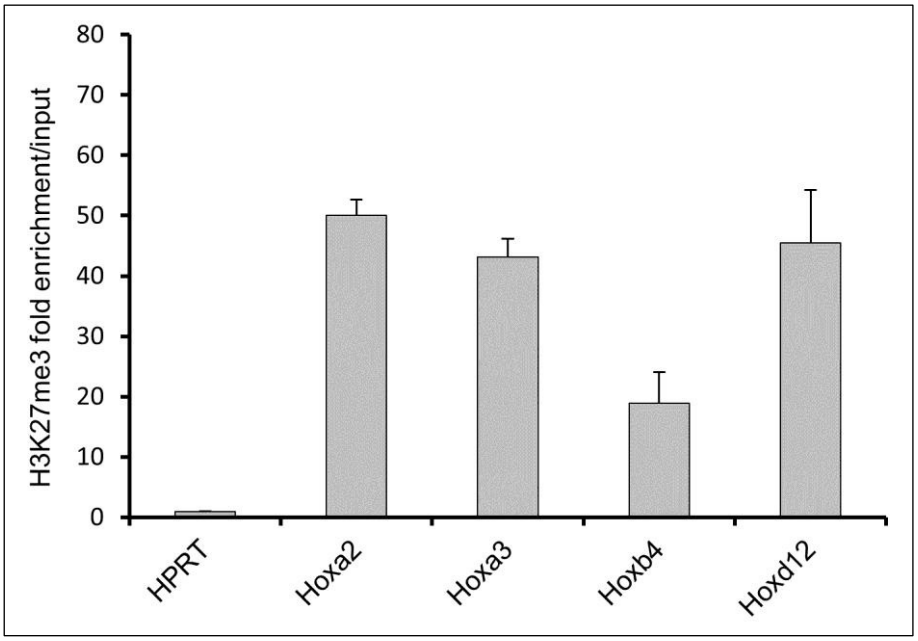

Figure 39: ChIP analysis of *Hoxa2*, *Hoxa3* and *Hoxb4*

Chromatin immunoprecipitation proofed the H3K27me3 occupancy around transcriptional start sites of *Hoxa2*, *Hoxa3* and *Hoxb4* and therefore regulation of these genes by Ezh2 in BA1 cells. ±S.D.

As expected from the literature (Boyer, Plath et al. 2006), a study showing the occupancy of H3K27me3 on the *Hox* gene cluster in ESCs, all three genes (*Hoxa2*, *Hoxa3* and *Hoxb4*) are target to H3K27me3 in wild-type BA1 cells as shown in Figure 39. These results demonstrate that the overexpression of Hoxa2, Hoxa3 and Hoxb4 in BA1 cells is due to the loss of H3K27me3 in Ezh2 cko embryos. And as already shown, these *Hox* genes are incompatible with skeletogenesis in CNCCs (Creuzet, Couly et al. 2002). This led us to the conclusion that we interfere with *Hox* gene expression, especially in the *Hox* negative domain (BA1 and rostrally). And especially in this domain the resulting overexpression of *Hox* genes is blocking the chondro- and skeletogenesis of CNCCs. Since CNCCs are the only subpopulation of NCCs which build up mesenchymal derivatives, the *Hox* gene overexpression has severe effects on the differentiation of CNCCs into skeletal elements of the craniofacial region.

5.11 Downregulated genes from the microarray

Table 6 shows transcripts that are highly significant downregulated in the microarray for at least 30%. Table 6 reveals the downregulation of ncRNA transcripts, like Dlx1as and Dlx6os1 and Dlx6os2. Further the table shows the already via immunohistochemistry shown downregulation of Collagens, like Col9a1 and Col2a1 (Figure 35). The collagens are thought to play a crucial role in chondrogenesis and it was already shown that Sox9 binds dirctly to the Col2a1 promoter (Bell, Leung et al. 1997). Further, transcripts of Sox5 and Sox6 are downregulated, as well. These genes have described roles in chondrogenesis, too (Yamashita, Miyaki et al. 2012). Another important gene family that was found to be downregulated in BA1 cells upon conditional knock-out of Ezh2 are the *Dlx* genes. These genes are known to

establish D-V axis in craniofacial development of BA cells, additionally to the A-P axis set up by *Hox* genes.

Gene symbol	ratio	p-value
Dlx1as	0.1378	6.09E-08
Dlx6os1	0.3655	4.85E-06
Dlx5	0.442	7.89E-07
Sox5	0.4543	3.12E-05
Msx2	0.4666	9.97E-06
Msx1	0.4899	6.56E-05
Dlx2	0.5101	1.96E-05
Dlx6os2	0.5167	0.000251
Sox5	0.5441	0.000629
Dlx3	0.5801	4.40E-05
Sox6	0.5803	0.000845
Col9a1	0.5849	0.000231
Dlx1	0.6309	5.15E-05
Dlx4	0.6631	0.000224
Col2a1	0.7064	0.000351

Table 6: List of selected downregulated genes

Some ncRNAs, Collagens, chondrogenic differentiation genes and many Dlx transcripts are highly significant downregulated in BA1 cells of ezh2 cko embryos when compared to control embryos. The Dlx genes are establishing the dorsal-ventral axis during mandibular development.

5.12 *Dlx* genes downregulation

The *Hox* genes might be crucial actors for the observed phenotype, but interestingly looking at highly significant downregulated genes in the microarray, we found many members of *Dlx* genes. The *Dlx* genes are a group of genes that establish the D-V patterning in BAs, additionally to the A-P axis that gets established by the *Hox* genes. Some *Dlx* genes are expressed along the D-V axis in BA1s and they show a nested expression, with declining expression levels towards distal (Kraus and Lufkin 2006). The microarray (Table 6) revealed a highly significant downregulation of Dlx5/6 and Dlx1/2 that we confirmed with q-RT-PCR. Further the downregulation of antisense transcripts was confirmed. Dlx1 a/s and Dlx6 os1 are ncRNAs read out from the *Dlx1* or *Dlx6* locus, respectively.

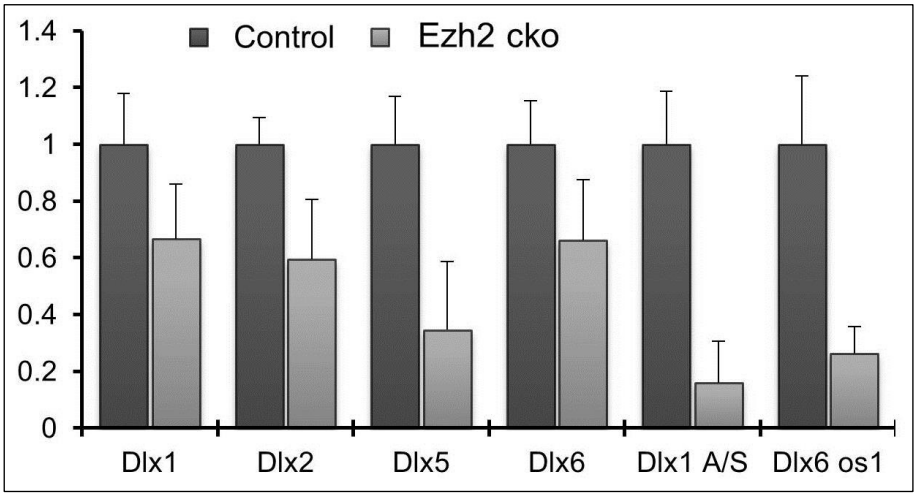

Figure 40: Dlx transcript downregulation upon Ezh2 loss in BA1 cells

q-RT-PCR showed the downregulation of Dlx genes and ncRNAs (Dlx a/s and Dlx6 os1) in Ezh2 cko BA1 cells when compared to control BA1 cells from E11.5 embryos. This proofs the downregulation of genes which establish the dorso–ventral axis. Bars ± SEM

6 Discussion

6.1 General

In my thesis we demonstrate that the PRC2 member Ezh2 is a key regulator of CNCC development and crucial for CNCCs to acquire a chondrogenic and osteogenic fate. *Ezh2* conditional ablation and concomitant loss of Ezh2-mediated H3K27 trimethylation in the NC resulted in agenesis of all NC-derived craniofacial structures. Surprisingly, however, *Ezh2* inactivation in the NC did not cause glial or neuronal defects in the PNS, indicating distinct requirements of epigenetic control mechanisms by different NC cell lineages. The only cells with NC origin capable of giving rise to skeletal elements of the face are located anterior to or in BA1 and BA2. Previously, it has been shown that the capacity of these cells for osteochondrogenesis is dependent on absence or downregulation of *Hox* gene expression (Minoux, Antonarakis et al. 2009). As shown here, conditional deletion of *Ezh2* in NC led to strong upregulation and misexpression of multiple *Hox* genes in BA1 cells. Furthermore, we identified *Hox* genes as direct targets of H3K27me3 dependent transcriptional repression in CNCCs. Thus, our findings reveal a crucial role of epigenetic gene silencing in regulating the formation of jaws and other craniofacial skeletal elements from NCCs.

6.2 Evolutionary aspects

The NC evolved in a step wise fashion. First appearances in cephalochordates and ascidians of neural crest like cells (NCLC) point to this. The expression of some markers at the right time and location that will in vertebrates become the gene regulatory network for the NC was already found in ascidians and cephalochordates (Gostling and Shimeld 2003; Jeffery 2006). Clearly, the NC was indispensable for development of jaws, permitting a mobile, predatory lifestyle. Evolutionary, this was first seen in gnathans (Gans and Northcutt 1983). The gene regulatory networks for NC development are complex and some of the mechanisms that ended up in NCCs induction were duplicated from older mechanisms. An example would be jaw development. Emigrating from the dorsal tip of the neural tube CNCCs get induced and specified in the rhombomeres. The rhombomeres by themselves are segregated and highly dependent on the correct spatial patterning of the A-P axis via *Hox* genes. CNCCs overtake their “Hox-status” upon emigration from the neural tube and retain it when populating the BAs, but the *Hox* gene expression status is adjustable (Trainor and Krumlauf 2001; Alexander, Nolte et al. 2009). In areas where osteogenesis follows the intermediate step of BA development *Hox* gene expression has to be repressed. BA1 and FNP CNCCs develop in the skeletal elements of the jaws and therefore harbor already at these earlier stages the osteogenic and chondrogenic precursors. A crucial repressor of *Hox* genes in *Drosophila* development are the *PcG* genes, among them *Ezh2*. In *Drosophila* the only *Hox* cluster (*Hox-C*) is responsible for A-P patterning and the expression patterns of the respective *Hox* genes determining the positional identity on the A-P axis. The A-P axis positioning system is the same in mammalians and regulated by *Hox* genes and *PcG* genes, as well (Schuettengruber, Chourrout et al. 2007). Howevert, additional functions of *Hox* genes in mammalian development are again

regulated by *PcG* genes. An additional role for *Hox* genes (e.g. *Hoxa2*) in mammalian development is to be a hyoid fate chooser in BA2 during NC development (Kanzler, Kuschert et al. 1998). Moreover, *Hox* genes (e.g. Hoxa2, Hoxa3 and Hoxb4) are known repressors of skeletogenesis (Couly, Grapin-Botton et al. 1998; Creuzet, Couly et al. 2002). The Hox code in CNCCs that populate the BA1 and the FNP has to be repressed, since these structures have to be devoid of *Hox* gene expression to acquire their skeletal fate. This repression is again mediated by *Ezh2* in mammalians as shown in this thesis. This could exemplify the "copying" of old systems to new functions. However, new regulatory elements have to evolve, a system directing the PRC complexes to silence the *Hox* gene expression in these cells again. The need of additional regulatory system of evolutionary conserved gene regulation systems might account for increasing amount of cis/trans regulatory elements in the genome during evolution (Sucena and Stern 2000; Trainor, Melton et al. 2003). Another possible regulation mechanism that might have evolved to control additional functions of already existing gene regulation systems could be ncRNAs. As shown in Figure 40 and Table 6, ncRNAs are downregulated in our Ezh2 cko BA1 cells. Dlx1 a/s and Dlx6 os1 are antisense transcripts from the *Dlx1* and *Dlx6* loci that are downregulated, as well. It is not completely clear what functional role ncRNAs have but mostly they are considered to play a gene regulatory role and are counted as epigenetic mode (Figure 8). During evolution their number increases. It could reflect the additional need of regulatory elements when overtaking conventional protein-gene interactions (Qu and Adelson 2012).

6.3 Early events of NC development and PNS formation is not affected

Early events of NC development including specification, migration and population of target structures could be a possible reason for the phenotype. After tracking NCCs with the *R26R-LacZ* reporter line it became obvious that NCCs expressing β-galactosidase are present in the supposed structures, like BAs and DRG in control and Ezh2 cko embryos. Embryos with comparable patterns of X-Gal positive cells are seen until E11.5 (Figure 19 and Figure 20). Therefore we concluded that the early events of NC development are not affected upon conditional knock-out of Ezh2. And surprisingly, loss of Ezh2 in NCCs did also not affect formation of neurons and glia in peripheral ganglia and nerves (Figure 25 and Figure24). This is somewhat different to the situation in the CNS, where Ezh2 controls the choice between proliferation and neuronal differentiation, as well as the timely fate switch from neurogenesis to gliogenesis (Hirabayashi, Suzki et al. 2009; Pereira, Sansom et al. 2010). In contrast, DRG and AG of *Ezh2* cko embryos contained normal numbers of sensory and autonomic neurons, respectively. Likewise, differentiation and timely appearance of satellite glia in ganglia and Schwann cells along peripheral nerves were not affected in *Ezh2* cko animals when compared to control embryos. Taken together, the generation and differentiation of neural lineages from NC cells are apparently not dependent on Ezh2-mediated epigenetic gene regulation. Interestingly, Shen and colleagues have previously reported lineage-specific requirements for Ezh2 during differentiation of mouse ESCs. *Ezh2* ablation in ESCs induced neural/ectoderm lineages at the expense of the mesodermal and endodermal differentiation programs. This suggests that the endodermal and mesodermal lineages are more susceptible to changes in Ezh2 activity than are neural lineages (Shen, Liu et al. 2008). Our findings are consistent with this hypothesis: Neural lineage formation remained unaffected upon inactivation of *Ezh2*

in the NC, whereas development of NC-derived mesenchymal progenitors, adopting a differentiation program similar to that of the mesoderm *in vivo,* was severely hampered in mutant mice.

An alternative explanation for unimpaired PNS development in *Ezh2* cko embryos is that in neural NC derivatives, Ezh1 activity might compensate for the loss of Ezh2 by catalyzing the methylation mark on H3K27, but fails to do so in cranial NC-derived MPCs. Others have previously demonstrated that upon loss of *Ezh2*, Ezh1 can overtake the catalytic function of Ezh2 for a particular subset of genes crucial for development (Boyer, Plath et al. 2006; Ezhkova, Lien et al. 2011). However, in the present study, conditional deletion of *Ezh2* was accompanied by a major loss of H3K27me3 in all NC derivatives analyzed, including PNS structures, suggesting that Ezh1 was not able to assume Ezh2 functions in a global manner in mutant NCCs. Nonetheless, we cannot rule out that a subset of genes crucial for neurogenesis remain occupied by H3K27me3 in *Ezh2* cko embryos due to Ezh1 compensation, allowing for normal PNS development. Furthermore, distinct epigenetic silencing mechanisms (e.g. H3K9 trimethylation and/or DNA methylation, in addition to H3K27 trimethylation) might have redundant functions in repressing gene expression during PNS development. Conceivably, however, loss of Ezh2 and H3K27me3 leads to gene de-repression also in the PNS, but these genes encode factors that apparently do not interfere with neuronal and glial differentiation programs when overexpressed.

6.4 NCSC to MPC transition and cell cycle properties of MPCs

During development, NCSCs originating in the cranial NC undergo a transition from NCSCs to MPCs, thereby losing neural potential and, at the same time, gaining the potential to produce mesenchymal lineages (John, Cinelli et al. 2011). This developmental switch is characterized by downregulation of the transcription factor Sox10 and concomitant upregulation of the transcription factor Sox 9. Sox9 has been implicated in craniofacial development (Mori-Akiyama, Akiyama et al. 2003), although its conditional ablation in the NC resulted in a much less severe phenotype than exhibited by *Ezh2* cko embryos. One possible explanation for the craniofacial phenotype observed upon *Ezh2* ablation may be failure of mutant NCSCs to give rise to MPCs. However, analysis of Sox9 and Sox10 expression levels did not reveal statistically significant changes upon *Ezh2* inactivation (Figure 28). Therefore we assumed that this differentiation step from NCSCs to MPCs might not be the cause of the phenotype, but another feature of stem/progenitor cells is their self-renewal capacity so we checked the cell cycle properties of MPCs in BA1, since Ezh2 is a well described modulator of cell cycle progression (Chen, Bohrer et al. 2010; Kaneko, Li et al. 2010; Zeng, Chen et al. 2011) and controls maintenance and proliferation of a variety of stem cell types in normal tissue and cancer (Bracken, Dietrich et al. 2006; Ezhkova, Pasolli et al. 2009; Suva, Riggi et al. 2009; Pereira, Sansom et al. 2010; Juan, Derfoul et al. 2011; Herrera-Merchan, Arranz et al. 2012; McCabe, Graves et al. 2012; Qi, Chan et al. 2012). In contrast, the number of NCCs emigrating from the neural tube and localizing to NC target structures was not subject to Ezh2 dependent regulation. In fact, NC-specific *Ezh2* ablation did not result in any changes in the cell cycle profile of NC-derived cells. These results indicate that the function of Ezh2 as a cell cycle modulator is tissue specific.

Moreover, our data reveal that Ezh2 exerts a major role in NCCs at relatively late stages of NC development, after NC specification and migration.

6.5 Ezh2 is required for craniofacial chondro- and osteogenesis

In contrast to NC specification, migration, population of target structures, PNS development and MPC establishment and cell cycle properties, Ezh2 activity is indispensable for osteochondrogenesis as already assumed from the observations of the phenotype at E12.5, E14.5 and E17.5 (Figure 21,Figure 22,Figure 23). Direct examinations on E14.5 and E17.5 Ezh2 cko embryos revealed the loss of almost all chondrogenic and skeletal elements that are derived from the NC (Figure 26 and Figure 27). Therefore one can assume that Ezh2 is crucial for the formation of the craniofacial skeletal elements originating from the NC. Not all skeletal elements are missing; this might be due to the NC specific knock-out of *Ezh2* in NCCs affecting just the craniofacial elements derived from this cell population. Other reports showed the mixed origin of the cranial bones of vertebrates in lineage tracing studies. Derived from mesodermal and NCCs our phenotype just blocks the skeletogenesis of NC derived bones (Jiang, Iseki et al. 2002; Gross and Hanken 2008). The first indications for the severe craniofacial phenotype are found already at early stages of chondrogenesis/skeletogenesis. E11.5 *Ezh2* cko embryos were impaired in chondrogensis as indicated by the lack of Col2a1 in mutant BAs (Figure 35). Additionally, other chondrogenic and osteogenic markers, such as Runx2, Osterix, and ALP, failed to be expressed by E11.5 BA1 cells upon *Ezh2* deletion (Figure 36). Therefore one can conclude that *Ezh2* inactivation prevents MPCs from acquiring an osteochondrogenic fate and, hence, producing cartilage and bone.

6.6 Ezh2 activity silences *Hox* gene expression in CNCCs

Craniofacial skeletal elements originating from the NC are exclusively produced from areas anterior to or located in BA1 and BA2, while NC cells posterior to these structures do not give rise to bone. Moreover, CNCCs that populate the BA1 and the FNP areas must have a *Hox* free ground pattern to undergo both forms of osteogenesis, endochondral and intramembranous ossification (Santagati and Rijli, 2003; Kanzler et al., 1998). BA2 cells express a single *Hox* gene, namely *Hoxa2*, but certain cells from this area undergo endochondral ossification, becoming the inner ear bones, incus, stapes and malleus. The chondrogenic domain preceding the endochondral ossification of these bones, named Reichelt's cartilage, is Hoxa2 free (Kanzler, Kuschert et al. 1998; Santagati and Rijli 2003; Minoux, Antonarakis et al. 2009). NCCs more caudal to BA2 are characterized by expression of several *Hox* genes and display a well-defined *Hox* gene expression code that varies according to the A-P position. These Hox factors suppress osteochondrogenesis, hence restricting this potential to anterior NC populations.

Large-scale analysis of ESCs has previously shown that Ezh2 occupancy, although widespread in the genome, is not global, but rather mostly associated with development related genes (Boyer et al., 2006). Among these, the *Hox* gene cluster turned out to be prominently regulated by Ezh2 in ESCs. Likewise, *Hox* genes are well-established targets of *PcG* regulation in *Drosophila* (Ringrose and Paro 2004; Sparmann and van Lohuizen 2006). Interestingly, our microarray analysis demonstrated that also in BA1 cells isolated from *Ezh2* cko embryos at E11.5 many *Hox* genes are heavily upregulated when compared to E11.5 control embryos. Therefore, we concluded that massive *Hox* gene misexpression might be a major, but not exclusively, cause of the craniofacial defects observed upon conditional knock-out of Ezh2 and concomitant loss of H3K27me3 in NC cells.

Several reports have suggested that erratic *Hox* gene expression causes impaired chondrogenesis and osteogenesis. For instance, in chicken embryos, *Hoxa2*, highly overexpressed in BA1 cells of *Ezh2* cko embryos is a well-known inhibitor of intramembranous bone formation from BAs (Couly, Grapin-Botton et al. 1998; Kanzler, Kuschert et al. 1998; Creuzet, Couly et al. 2002). Massip and colleagues have shown that ectopic expression of Hoxa2 in chondrocytes results in impaired differentiation, as demonstrated by the lack of Col2a1, and prevents cartilage formation (Massip, Ectors et al. 2007). In BA2, Hoxa2 is a promoter of the hyoid fate of mesenchymal CNCCs and restricts the chondrogenic domains that arise during endochondral ossification (Pasqualetti, Ori et al. 2000). Hoxa2 gain-of-function in BA1 cells results in a duplication of BA2 skeletal elements in *Xenopus* (Pasqualetti, Ori et al. 2000), whereas Hoxa2 loss in BA2 in mice leads to a mirror homeotic transformation of BA1 skeletal elements (Rijli, Mark et al. 1993). Importantly, Hoxa2 in BA2 prevents osteogenesis by blocking its main effector Runx2. In addition, Hoxa2 is also involved in cartilage patterning until E11.5 by an independent mechanism that involves different cofactors. Conditional gene inactivation further revealed that subpopulations of CNCCs require Hoxa2 at defined time points for proper BA patterning (Santagati, Minoux et al. 2005). Hoxc8 is another factor that prevents endochondral differentiation by maintaining chondrocytes in a proliferative state and inhibiting chondrocyte hypertrophy (Yueh, Gardner et al. 1998). Furthermore, Hoxa9 is known to be a direct repressor of the earlier bone inducer osteopontin in lung epithelial cell lines (Shi, Bai et al. 2001). Finally, Hoxa3 and Hoxb4 are also well known inhibitors of osteogenesis in NC precursor cells. When individually overexpressed in NC derivatives of chicken embryos, Hoxa3 expression results in formation of rudimentary nasofrontal bud derivatives, whereas Hoxb4 impairs terminal differentiation of BA1 derivatives such as the proximal bones (Couly, Grapin-Botton et al. 1998; Creuzet, Couly et al. 2002). However, when jointly

overexpressed, Hoxa3 and Hoxb4 block skeletogenesis to a similar extent than ectopic Hoxa2 expression.

6.7 Dlx genes and D-V axis

Additionally to the A-P axis specification of the *Hox* genes and their incompability with skeletogenesis, the *Dlx* genes determine the D-V axis in BAs. Studies on mice showed that the loss of Dlx5/6 and Dlx1/2 resulted in jaw and skull malformations and the *Dlx* genes are described to play a role in endochondral ossification, especially Dlx5/6. *Dlx* genes are expressed in a nested fashion in BA1, Dlx1/2 broadly and Dlx5/6 becoming progressively more distally restricted (Qiu, Bulfone et al. 1997; Acampora, Merlo et al. 1999; Depew, Lufkin et al. 2002; Kraus and Lufkin 2006). Since agnathans express at least one *Hox* gene in the mandibular arch and gnathans do not and further the Dlx expression in the branchial arches is nested, these events were suspected to be critical steps in the evolution of jawed vertebrates (Cohn 2002; Trainor, Melton et al. 2003). Our microarray revealed a downregulation of *Dlx* genes (Figure 40; Table 6), additionally to the *Hox* gene overexpression (Table 5). Since we were conditionally knocking out Ezh2, a repressor of gene expression, the downregulation of *Dlx* genes is more likely a secondary effect. Possibly, the expression of *Hox* genes taken over by CNCCs from the rhombomeres might occur earlier leading to the assumption that these two gene effector cascades could influence each other. An assumption would be that expression of *Hox* genes in BA1 cells prevents the nested expression of *Dlx* genes that is necessary to establish the D-V axis in BA1 cells during jaw development. Disturbing the D-V axis position in BA1 cells results in a phenotype that partially resembles to a lesser extend the observed phenotype in our Ezh2 cko embryos. Therefore it is likely that the *Dlx* specification of the D-V axis could contribute to our phenotype.

6.8 Conclusion

All these events indicate that NC precursors are specified for osteochondrogenesis in a temporally controlled manner, and that finely tuned regulation of *Hox* gene expression is crucial for this process. In BAs of *Ezh2* cko embryos, high and ectopic expression of the *Hox* genes mentioned above to suppress osteo- and chondrogenesis (Hoxa2, Hoxa3, Hoxb4, Hoxa9, and Hoxc8), as well as of many other *Hox* genes, is associated with inhibition of chondrogenic and osteogenic gene expression programs and a virtually complete blockage of cartilage and bone formation from NCCs. In contrast, Ezh2 inactivation in NC does not affect neural lineage formation, which is in agreement with a previous study reporting undisturbed PNS development upon combined Hoxa2, Hoxa3 and Hoxb4 overexpression in CNCCs (Creuzet, Couly et al. 2002). Thus, Ezh2-mediated H3K27 trimethylation plays a lineage-specific role in NC cells, repressing *Hox* genes in anterior NC subpopulations and thus allowing osteochondrogenesis in these cells. Clearly, region-specific *Hox* gene regulation and the potential to form skeletal structures must involve other factors as well, given that Ezh2 and H3K27me3 are broadly expressed also in more posterior NC cells that *in vivo* do not form cartilage and bone. These factors include intrinsic cues distinguishing CNCCs from more caudal NC cells, as e.g. suggested by clonal assays in cell culture or by *in vivo* manipulation of signaling pathways regulating neural and osteochondrogenic fates, respectively (Buchmann-Moller, Miescher et al. 2009; Calloni, Le Douarin et al. 2009; John, Cinelli et al. 2011). The nature of these cues and their potential functional interaction with epigenetic regulators including Ezh2 remains to be determined. Moreover, this study raises the intriguing question of whether and how epigenetic control of osteochondrogenesis in NCCs was implicated in the evolution of jawed vertebrates.

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8 Curriculum vitae

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